











# MICRO-ORGANISMS AND FERMENTATION.

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### TRANSLATOR'S PREFACE.

A TEXT-BOOK written by one of the foremost exponents of the honoured Danish School of Micro-Biological Research, and by a pioneer of world-wide reputation in the industrial application of selected types of yeast, is certain of a welcome from English readers.

In comparison with the enormous output of works on the organisms of disease, little has yet been published in English on the technical applications of Micro-Biology. This book covers ground which is not fully surveyed in any existing treatise. The necessity of embodying the results of ten years' research has led to such a mass of additions and alterations in the last English edition that this must be regarded as a new work. It is based on the fifth German edition.

S. H. DAVIES.

YORK,  
*December, 1910.*

## P R E F A C E.

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THE first edition of this work<sup>1</sup> was issued in the form of a text-book in 1886. It was the first attempt to express the biological significance of the science of fermentation and of the fermentation industry, a field where the chemical point of view had hitherto prevailed.

I was induced to give this form to my work by the fact that in 1881 I had established an institute in which my first aim was to treat the problems of the fermentation industries from a micro-biological point of view. This necessitated a short course for technologists and chemists who wished to study the science of fermentation on new lines, and as both older and younger students were attracted to my laboratory, the subject-matter had to be arranged so that the book could serve as a guide, even to those who had no special preliminary knowledge. During my co-operation with E. C. Hansen in that early period, the principles of the practical application of pure cultures were broadly outlined, and accordingly an explanation of the principles underlying the resulting technical reforms formed the essence of the treatise. The new editions which appeared in rapid succession, showed that the attempt had been successful. At the same time, I had the good fortune to introduce into breweries in many countries properly selected pure cultures of bottom yeasts, following the precedent of E. C. Hansen, who shortly before had introduced similar yeasts into the Carlsberg Brewery in Copenhagen. At this early stage, I successively described pure cultures of yeast types which are used in other branches of the fermentation industry, and I introduced them into top-fermentation breweries, as well as into distilleries, yeast factories, fruit-wine factories, etc. Thus a beginning was made in the application of this new principle to new fields of industry. Consequently, to keep pace with these developments, together with the results of research in the science of fermentation, my book had to be so largely modified that each new edition became to some extent a new book, whilst the growing mass of material considerably increased the size of the book. Having to keep within certain limits, I was reluctantly compelled to

leave various problems undiscussed. On the other hand, repetition could not always be avoided for the sake of young students, when similar subjects were discussed in different sections.

Among the many new questions demanding fuller treatment in this, the fourth English edition, I may specially mention the fermentation of milk and other dairy products; the enzymes, and the conditions of yeast nutriment. As regards the technical part of the work, a new section deals with methods of cleansing and disinfection in the fermentation industry, and the section dealing with the application of pure cultures to the various branches of the fermentation industry has been considerably enlarged. In its present form the book will, I hope, serve as a manual, not only to zymo-technologists, but also to analysts and physiologists.\*

For convenience, the old names of yeast species have been retained along with the new, in the systematic treatment given in the fifth chapter.

As the work is concerned with the micro-organisms of the fermentation *industry*, due regard has been paid to the practical application of research work, and the description of both useful and injurious species has received special attention. The classification of the yeasts has been partially based on the same requirements. The description of each species emphasises those characteristics that are of special importance to the industry.

I am glad to take the opportunity of expressing my thanks to the head of the students' department in my laboratory, Herr J. C. Holm; the systematic part of Chap. v., together with the very full bibliography, are essentially his work.

Finally, I wish to express my warm thanks to many authors who have kindly sent me reprints of their published works. I regret that the limited extent of my book has in many cases prevented me from making use of valuable publications.

COPENHAGEN, January, 1909.

ALFRED JÖRGENSEN.

\* A short description of the most important practical conditions found in the fermentation industry and in the laboratory is to be found in my short work, *Practical Management of Pure Yeast*. London, 1903.

## CONTENTS.

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### CHAPTER I.

#### Microscopical and Physiological Examination.

	PAGES
1. Microscopical Preparations; Staining, and Microscopical Examination,	1-10
2. Biological Research; the Microscope; Moist Chambers, . . . . .	10-13
3. Sterilisation, . . . . .	13-31
(a) Sterilisation of Glass and Metal Articles, . . . . .	14-15
(b) Sterilisation of Liquids and Solid Nutritive Substrata, . . . . .	15-31
4. Disinfection in Practice, . . . . .	31-36
5. Flasks, . . . . .	36-40
6. Nutritive Substrata, . . . . .	40-43
7. Preparation of Pure Culture, . . . . .	43-55
(a) Physiological Methods, . . . . .	45-47
(b) Dilution Methods, . . . . .	47-55
8. Counting the Yeast Cells, . . . . .	55-58

### CHAPTER II.

#### Biological Examination of Air and Water.

Introduction, . . . . .	59-60
Air Analyses—	
Miquel's Methods and Results, . . . . .	60-62
Methods of Hesse, Hueppe, von Schlen, Frankland, Miquel, Petri, and Ficker, . . . . .	62-63
Sampling Air, . . . . .	64
Miquel's Criticism of the Gelatine Process, . . . . .	64
E. C. Hansen's Researches on Air, . . . . .	65-69
Saito's Researches, . . . . .	69
Water Analyses—	
Holm's and Jörgensen's Examination of Water, . . . . .	69-71
Examination of Ice, . . . . .	71
Methods of Hansen, Wichmann, Lindner, and Jörgensen, . . . . .	71-75

## CHAPTER III.

## Bacteria.

	PAGES
Forms, . . . . .	76-78
Anatomical Structure, Colouring Matters, Phosphorescent Bacteria, . . . . .	78-80
Chemical Composition, . . . . .	81
Nutrition, . . . . .	81
Anaërobiosis, . . . . .	82
Locomotion, . . . . .	83
Propagation, Spore-Formation, . . . . .	83-85
Influence of Temperature, Light, Pressure, Antiseptics, and Mechanical Vibration, . . . . .	85-87
Classification, . . . . .	88-92
1. Acetic Acid Bacteria, . . . . .	92-105
2. Lactic Acid Bacteria, . . . . .	106-125
3. Butyfic Acid Bacteria, . . . . .	125-132
4. Bacteria Fermenting Cellulose, . . . . .	132-133
5. Alcohol-forming Bacteria, . . . . .	133-135
6. Kephir, Koumiss, Mazun, Leben, Yoghourt, Ginger-Beer, . . . . .	135-141
7. Slime-forming Bacteria, . . . . .	142-151
8. Bacteria with Inverting, Diastatic, and Proteolytic Enzymes, . . . . .	151-153
9. Sarcina, . . . . .	153-160
10. The Fermentation of Tobacco, . . . . .	160-161
11. Iron and Sulphur Bacteria. Nitrifying Bacteria, . . . . .	162-164

## CHAPTER IV.

## Moulds.

Introduction, . . . . .	165-166
Anatomical Structure, . . . . .	167-171
Classification of the Fungi, . . . . .	171
Nutritive Physiology, . . . . .	171-175
Action of External Influences, . . . . .	175-178
Chemical Constituents; Enzymes, . . . . .	178-183
Botrytis cinerea (Sclerotinia Fuckeliana), . . . . .	183-187
Penicillium glaucum, . . . . .	187-191
Aspergillus, . . . . .	191-197
Mucor, . . . . .	197-210
Monilia, . . . . .	210-214
Oidium lactis, . . . . .	214-216
Fusarium, . . . . .	217
Chalara, . . . . .	217-218
Dematium, . . . . .	218-220
Cladosporium, . . . . .	221-222
Oidium (Erysiphe) Tuckeri, . . . . .	222-223
Peronospora, . . . . .	223-224

## CHAPTER V.

## Yeasts.

	PAGES
Introduction, . . . . .	225
Nutrition of Yeasts, . . . . .	225-229
Theories of Fermentation, . . . . .	229-255
The Enzymes of Yeast, . . . . .	255-256
The Action of the <i>Saccharomyces</i> and similar Fungi on Carbohydrates and other Constituents of Nutritive Liquids—Diseases in Beer, . . . . .	256-266
The Products of Alcoholic Fermentation, . . . . .	267-269
Auto-Fermentation, . . . . .	269-270
Fermenting Power, Fermentative Energy, Raising Power, . . . . .	270
The Biological Relationship of Yeast, . . . . .	270-280
Variations in the <i>Saccharomyces</i> , . . . . .	280-289
Morphology and Anatomy of Yeast Cells, . . . . .	290-313
Yeast Deposits, . . . . .	290-292
Film Formation, . . . . .	292-297
Cultures on Solid Substrata, . . . . .	297-298
Structure and Character of Yeast Cells, . . . . .	298-312
Ascospore Formation, . . . . .	302-310
Analysis of Yeast, . . . . .	310-313
I. <i>Saccharomyces</i> , . . . . .	314-385
Classification of the <i>Saccharomyces</i> , . . . . .	315-317
1. Species used Industrially (Culture Yeasts), . . . . .	317-344
(a) Brewery Yeasts, . . . . .	317-341
Classification, . . . . .	318-319
The use of Pure Bottom Yeasts in Practice, . . . . .	319-320
Carlsberg Bottom Yeasts I and 2, . . . . .	320-323
Low-Fermentation Yeasts described by Will, . . . . .	324
“Saaz” and “Frohberg” (P. Lindner), . . . . .	325
Low-Fermentation Yeasts described by Schönfeld and Rommel, . . . . .	325
<i>Saccharomyces cerevisiae</i> or <i>Sacch. cer.</i> I. (Hansen), . . . . .	326-328
Top-Fermentation Yeasts described by Jørgensen and Holm, . . . . .	329-339
Top-Fermentation Yeasts described by Regensburger, . . . . .	339-341
The use of Pure Top-Fermentation Yeasts in Practice, . . . . .	341
(b) Distillery and Press Yeasts, . . . . .	342-343
(c) Wine Yeasts, . . . . .	343-344
2. Yeasts not yet applied Industrially, . . . . .	345-385
<i>Saccharomyces Pastorianus</i> or <i>S. Past.</i> I., . . . . .	345-347
<i>S. intermedium</i> or <i>S. Pastorianus</i> II., . . . . .	347-349
<i>S. validus</i> or <i>S. Pastorianus</i> III., . . . . .	349-351
<i>S. ellipsoideus</i> or <i>S. ellips.</i> I., . . . . .	351-353
<i>S. turbidans</i> or <i>S. ellips.</i> II., . . . . .	354-355
<i>S. Willianus</i> , . . . . .	355
<i>S. Bayanus</i> , . . . . .	356

## CONTENTS.

	PAGES
<i>S. Logos</i> , . . . . .	356-357
<i>S. thermantitonum</i> , . . . . .	357
<i>S. Ilicis</i> , . . . . .	358
<i>S. Aquifolii</i> , . . . . .	358
<i>S. Vordermanni</i> , . . . . .	358
<i>S. Saké</i> , . . . . .	359
<i>S. Batatae</i> , . . . . .	359
<i>S. cartilaginosus</i> , . . . . .	359-360
<i>S. multisporus</i> , . . . . .	360
<i>S. mali</i> Risler Kayser, . . . . .	360
<i>S. Marxianus</i> , . . . . .	360-361
<i>S. exiguis</i> , . . . . .	361-362
<i>S. Jörgensenii</i> , . . . . .	362
<i>S. Zopfii</i> , . . . . .	362-363
<i>S. Bailii</i> , . . . . .	363
<i>S. hyalosporus</i> , . . . . .	363
<i>S. Rouxi</i> , . . . . .	364
<i>S. Soya</i> , . . . . .	364
<i>S. mali</i> Duclaux Kayser, . . . . .	365
<i>S. unisporus</i> , . . . . .	365
<i>S. flava lactis</i> , . . . . .	365
“Levure de sel” <i>a</i> , . . . . .	365-366
<i>S. Hansenii</i> , . . . . .	366
<i>S. minor</i> , . . . . .	366
<i>Pichia membranæfaciens</i> or <i>S. membr.</i> , . . . . .	366-368
<i>Willia anomala</i> or <i>S. anomalus</i> , . . . . .	368-370
<i>Willia Saturnus</i> or <i>S. Sat.</i> , . . . . .	370
<i>S. acidi lactici</i> , . . . . .	370-371
<i>S. fragilis</i> , . . . . .	371-372
<i>Zygosaccharomyces Barkeri</i> , . . . . .	372-374
<i>Saccharomyces Ludwigii</i> or <i>Sacch. L.</i> , . . . . .	374-377
<i>Schizosacch. or Sacch. comesii</i> , . . . . .	377
<i>Schizosacch. octosporus</i> , . . . . .	377-379
<i>Schizosacch. Pombe</i> , . . . . .	380-381
<i>Schizosacch. mellacei</i> , . . . . .	381-383
<i>Saccharomycopsis</i> or <i>Saccharomyces guttulatus</i> , . . . . .	383-384
<i>Saccharomycopsis capsularis</i> , . . . . .	384-385
II. Budding Fungi without Spore-Formation, . . . . .	385
<i>Torula</i> , . . . . .	385
Hansen's species, . . . . .	385-388
<i>Torula Novaæ Carlsbergia</i> , . . . . .	388
<i>Torula a</i> = <i>T. Holmii</i> , . . . . .	388
<i>Torula A</i> and <i>B</i> , <i>Schiönnig</i> . . . . .	389-391
<i>Torulas</i> described by <i>Will</i> , . . . . .	391-392
<i>Torulas</i> described by <i>van Hest</i> , . . . . .	392-393
<i>Torulas</i> described by <i>Meissner</i> , . . . . .	393
<i>Torula colliculosa</i> , . . . . .	393

	PAGES
Torulas described by Adametz, . . . . .	393-394
“Salt Yeast,” . . . . .	394
Torula epizoa, . . . . .	394
“Levure de sel” $\beta$ and $\gamma$ , . . . . .	394-395
Sacch. brassicae, . . . . .	395
Torula (Awamori), . . . . .	395
Torula b = mucilaginosa, . . . . .	395-396
Torula c = cinnabarina, . . . . .	396 397
Torula in Beer or Green Malt, . . . . .	397-398
Blastoderma salmonicolor, . . . . .	398
Mycoderma humuli and rubrum, . . . . .	398
Torula in Milk and Must, . . . . .	398
Black Yeast, . . . . .	398
Torula Yeasts fermenting Lactose, . . . . .	399
Sacch. lactis, . . . . .	399-400
Sacch. Kephyr and Tyrocola, . . . . .	400
Lactomyces inflans caseigrana, . . . . .	400
Torula in Butter, Cheese and Milk, . . . . .	400-401
Torula amara, . . . . .	401
Saccharomyces apiculatus, . . . . .	402-407
Mycoderma cerevisiae and vini, . . . . .	407-413

## CHAPTER VI.

## The Pure Culture of Yeast on a Large Scale.

Industrial Application, . . . . .	414
Propagating Apparatus, . . . . .	415-418
Despatch of Pure Cultures, . . . . .	419
Preservation of Pure Cultures, . . . . .	420
 BIBLIOGRAPHY, . . . . .	 421
INDEX, . . . . .	481



# MICRO-ORGANISMS AND FERMENTATION.

## CHAPTER I.

### MICROSCOPICAL AND PHYSIOLOGICAL EXAMINATION:

#### 1. Microscopical Preparations, Staining, and Microchemical Examination.

THE Microscope will always be the chief means for investigating micro-organisms, for these are, as individuals, almost always invisible to the naked eye.

The microscope is made up of a mechanical and an optical part. The mechanical part, or **stand**, consists of the **foot**, the **stage**, the **tube** carrying the lenses, and the **adjustment** for regulating the distance between the lens and the object lying on the stage. The adjustment is partly "coarse" (a screw engaging in a toothed gear attached to the tube) whereby the tube can be rapidly raised or lowered, partly "fine" (a finely cut screw) by means of which the tube can be gradually raised or lowered, after finding the object with the coarse adjustment. The tube commonly consists of two telescoping parts. A table, which usually accompanies the instrument and gives the scale of magnification, records the corresponding **length of tube** either in millimetres (usually 180 mm.) or in inches (usually 10").

The optical part consists of the **lenses** and **illuminating apparatus** (a mirror and Abbé condenser).

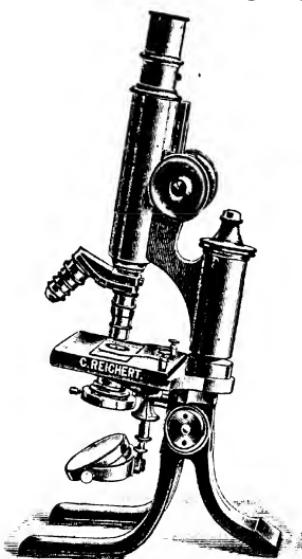


Fig. 1.

The lenses form the most important part of the microscope; the system turned towards the eye is called the eyepiece, and that turned towards the object is called the objective.

When a bundle of parallel rays of light strikes a convex lens, the rays are refracted and collect at a point on the other side of the lens, called the **focus**. The distance between this point and the lens is called the **focal distance**. If a small object is placed on the stage at a slight distance beyond the focal length of the lens and illuminated by the mirror, the rays passing through the lenses of the objective will not be parallel, but will diverge, and so form a **magnified** image of the object. The smaller the focal length, the greater the magnification. This real inverted and magnified image formed by the objective, which must fall exactly in the focus of the eyepiece, is seen through the latter as an imaginary,

magnified image of the picture formed by the objective. The whole magnification secured by the microscope is, therefore, a product of the magnification due to the objective and that due to the eyepiece. The magnification is always expressed as a linear and not as a quadratic dimension—*i.e.*, the relationship between the length of a line as seen through the microscope and its length as seen by the naked eye.

From any given point of the object on the stage of the microscope a bundle of rays may pass through the lens. The angle which the outermost rays of the bundle form is called the **angular aperture**. It is not customary to allow all the rays of the angular aperture to pass through the system of lenses which make up the objective. A larger or smaller number of the peripheral rays are excluded by means of diaphragms consisting of metal discs with round apertures, which are of various sizes, and fit into the opening on the stage. In this way the actual angular aperture of the objective is reduced. The peripheral rays would give an indistinct picture, but the picture would, on the other hand, lose in clearness if too many of the outer rays were excluded.

For this reason the objective is so constructed that the aperture shall be as great as possible. An expression for the value of the latter is given by the numerical aperture (the sine of half the angular aperture multiplied by the index of refraction of the medium in front of the lens—air, water, oil, etc.).

When the rays of light are refracted in the objective some of them are immediately split up into the component coloured rays, and the image shows coloured edges (**chromatic aberration**). To avoid this the objective is made up of several different lenses prepared from various kinds of glass (crown glass, flint glass) which possess different refractive powers. It is thus possible to prevent any indistinctness of outline.

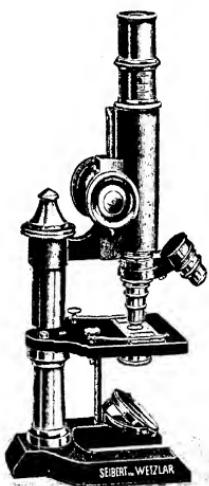


Fig. 2.

To secure greater magnification a system of **immersion** is used, the space between the front lens of the objective, which is made of crown glass, and the cover glass being filled with a strongly refractive medium, water or oil. The immersion is **homogeneous** if, as is often the case, the oil has the same index of refraction as the cover glass and the front lens. In this way an increase of the numerical aperture is secured, and, therefore, a greater **resolving power**—the limit of definition for the smallest perceptible details.

The **eyepiece** consists of two lenses, the upper, which comes into direct contact with the eye, and the lower, called the **collective lens**, which collects the rays of light so that the **field of vision** is reduced, and is, therefore, more easily surveyed. Between the two lenses a diaphragm is inserted in order to further reduce the field. The collective lens is also of importance in securing, along with the upper lens, the exclusion of the coloured edges of the microscopical picture.

The greater the curvature of the lenses in the eyepiece, the more do they magnify the image projected by the objective, which at the same time becomes darker and less distinct. To obtain a well-lighted field when working with high magnifications, it is necessary to use strong objectives and weak eyepieces.

It is well known that the lenses of the human eye alter their shape according to the distance of the objects that are under observation. They can accommodate themselves, and, by projecting, shorten the focal length, and thereby produce a sharp image on the retina. On the other hand, by a reduction of the convexity the focal length is increased, and thus a clear image of distant objects may be thrown on to the retina. If the eye is short-sighted or long-sighted, the tube of the microscope must be set to suit the focal length of the eye, and the size of the image will differ for abnormal eyes. The normal focal distance is considered to be 250 mm., and tables of magnification are based on this. The actual magnification for any individual eye must be established by a special calculation with the help of a glass micrometer.

The **illumination** of the object is secured by a mirror placed below the stage which is capable of movement in all directions, so that the object can receive direct or oblique illumination, and so that the mirror can be fixed at different distances from the object. For low powers the plane side of the mirror is used, for high powers the concave side. With ordinary magnifications it is of importance to secure suitable illumination, as the eye soon tires if the light is too strong. Instead of the usual diaphragm in the stage, an **iris diaphragm** may be used, enabling the aperture to be reduced or enlarged by means of a number of sickle-shaped leaves sliding over each other. To give illumination over a large surface (e.g., in the examination of coloured substances), a combination of lenses known as a **condenser** is introduced between the mirror and the preparation. When studying an object, the separate parts of which can only be distinguished by differences in their refractivity, a narrow bundle of rays must be used, and this is secured by placing a diaphragm with a smaller opening in the aperture of the stage.

The microscopical examination of the organisms of fermentation throws light upon their size, form, colour, the

refractive indices of different parts of the cell, and, generally, of all those conditions which are the object of **morphological** research.

As we are dealing with living forms, we can only arrive at a real knowledge of them by studying their life conditions, through biological and physiological research.

**Biological** research is concerned with the investigation of life phenomena under the conditions existing in nature ; thus, such conditions as the distribution of single species ; their occurrence ; the numbers present in different localities at different seasons ; their sensitiveness to light, to heat, to the moisture of the atmosphere, etc.

**Physiological** research has for its object the study of the life history of the organism, the conditions of nourishment and propagation. It is also concerned with the different kinds of fermentative activity, so far as these can be established by studying the influence of organisms on the liquids in which they are growing, and with the nature of the substances or forces causing fermentation (enzymes). Specially constructed apparatus is available for such investigations, and many of these lines of research are closely allied with chemical studies.

One essential condition of any exact investigation into the life history of micro-organisms must be secured—the certainty that we are working with a single cell or with one vegetation, consisting of a single species, and, therefore, derived from one cell. We shall see in the following pages how the technique has been slowly developed, and how this goal has been reached, as the result of many scientific and technical attempts to prepare absolutely **pure cultures**.

For the ordinary examination of yeasts and moulds, a clear magnification of 600 suffices. For the examination of the fine details of these organisms and of bacteria, higher powers are required. Although an immersion lens is of great service, it is not essential for ordinary technical work.

It is of real importance that the organisms of fermentation should be examined, as much as possible, in a living state, and either in a drop of water or of a liquid in which they have been growing. The drop is placed on an object glass,

and spread out to form a thin layer, by placing a cover glass on top of it, or else the drop may be placed inside a moist chamber (described later) in which the growth and propagation of the cells can be followed. For instance, by such observations of the living cell, the development of spores in the yeast cell may be observed, and the difference in shape of the ripe spore in individual species, the thickness of the wall of the mother-cell, etc., may be noted.

Certain characters, however, of the detailed construction of these organisms can only be detected by the use of special drying and staining methods. To do this, the cells are subjected to a thorough treatment with concentrated dyes, some of a poisonous character. They are killed, and the possibility exists that the characters brought out by staining may differ somewhat from those of the living cell. Drying may also modify the length and breadth of the bacteria. On the other hand, the staining process has explained many phenomena which were not apparent by observation of the living cell.

Dilute dyes (e.g., eosin, methylene blue) are used in the technical examination of yeasts to obtain an idea of the proportion between dead and living cells in a vegetation, since the dead cells alone absorb the dye.

As an example of a method of staining, we may instance the treatment to which yeast cells are subjected in order to observe the cell-nucleus and its subdivision. Hoffmeister proceeds as follows :—The young, vigorous yeast growth is washed several times with distilled water, and then before the actual staining it is subjected to the process of fixing, according to one of the recognised methods. For instance, the yeast is stirred up with Rath's solution (consisting of a litre of a concentrated, aqueous solution of picric acid, together with 4 c.c. of glacial acetic acid and 1 gramme of osmic acid); the cells are thus coloured yellow. After allowing the mordant to react for 24 hours, the cells are again washed with water, spread out in a thin layer on a cover-glass, and allowed to dry. The preparation is then treated according to Heidenhain's method. The cover-glass preparation is allowed to float in a Petri dish, on the surface of a solution containing 2·5 per cent. of iron alum; after 6 to 24 hours the cover-glass is washed once with

water, and then placed in an aqueous hæmatoxylin solution. After a further 24 hours the cells, as seen under the microscope, are stained deep black. They are then treated for a few minutes with a 0.25 per cent. solution of iron alum, after which the yeast cells appear colourless with violet or greyish-black cell nuclei. The preparation is mounted in undiluted glycerine. One of the more advanced of recent workers in this field, A. Guilliermond, makes use of the method of staining just described to prove the presence of the cell-nucleus in the yeast cells, but for fixing he prefers picroformol (*Bouin*).

To prove the presence of the fine hairs, which serve the bacteria as organs of movement, the flagella or cilia, which can seldom be detected by direct microscopical examination of the living bacteria, the following method (*Löffler*) is adopted:—A small quantity of a very young growth of bacteria (developed for five to eight hours in an incubator) is placed in a drop of water—the ordinary supply is preferable to distilled water—and the contents of this drop are divided amongst a number of drops of water, placed on a series of carefully cleaned cover-glasses. They are air-dried, and are then passed through a flame in order to fix the bacteria. Care must be taken that the preparation is not heated too strongly. The simplest means of avoiding this is to hold the cover-glass between the fingers, and not to heat it more strongly than they can bear. A large drop of a mordant is now spread over the heated cover-glass. The mordant, which is applied to render the bacteria absorbent to the actual stain, consists of 10 c.c. of tannic acid solution (20 per cent.) mixed with 5 c.c. of a cold saturated ferrous sulphate solution and 1 c.c. of a saturated aqueous or alcoholic fuchsine solution. The cover-glass is warmed for about half a minute until steam is given off, but violent boiling must be avoided. The preparation is washed with a powerful stream of distilled water, and afterwards with absolute alcohol until the cover-glass is clear, and only the spot on which the water drop has been evaporated appears cloudy. The staining fluid is now poured over the surface of the cover-glass. It consists of a neutral saturated fuchsine solution in aniline. The preparation is warmed again for a minute until steam rises, washed with a stream of water,

and is then ready for examination. It should be noted that all motile bacteria do not show their flagella when they are treated with a mordant of the above composition. One must proceed experimentally, for some bacteria require a mordant to which a few drops of a 10 per cent. soda solution have been added, whilst others require an addition of sulphuric or acetic acid in place of soda. Löffler found that several of the acid-forming bacteria require an alkaline mordant, whereas a number of alkali-forming organisms require an acid mordant. By such careful means beautiful pictures have been obtained, which show that bacteria are supplied with these organs of movement arranged in various ways; they often cover the whole surface of the cell. A similar treatment brings out clearly the coating of slime which, for instance, surrounds the cells of acetic acid bacteria, but is invisible in an ordinary microscopical examination.

In a few cases staining has proved of value in determining the species; this is the case with the acetic acid bacteria investigated by E. C. Hansen, *Bacterium aceti*, *B. Pasteurianum*, and *B. Kützingianum*. Staining is most readily done by treating a vigorous growth with an aqueous solution of iodine in potass. iodide, or an alcoholic solution of iodine. The slime surrounding the cells of *B. aceti* is coloured yellow, whereas that of the other two species is coloured blue. The latter reaction is brought out more clearly when the slime is forced out to the sides by pressure on the cover-glass. Blue coloured flecks are thus formed, while the cells themselves are either colourless or yellow. A similar blue coloration of the spores of *Saccharomyces mellacei* with iodine has also been demonstrated by J. C. Holm.

Photographs of micro-organisms are now frequently employed. The preparation may be first stained, for instance, by Löffler's method, to bring out the characteristics more sharply, and to remove foreign bodies. Whilst the usual sketches of microscopical preparations are always more or less diagrammatic, omitting everything except the special characters it is desired to emphasise, micro-photography provides a more correct representation of the object, and has a further incidental advantage. It is well known that

the photographic plate is sensitive to certain chemically active rays of light which cannot be detected by the human eye, so that photography can reproduce certain characters of the preparation which would be entirely missed by direct observation. It may be anticipated that the new method of photographing by ultra-violet light will bring about notable advances, for it will thus be possible to distinguish a greater number of fine characteristics and preparations otherwise colourless will appear to be composed of differently coloured parts.

The ultra-microscope that has just been applied to technical purposes makes it possible to distinguish small details that are invisible with all earlier optical devices. Its design is based upon a special method of lighting, discovered by Siedentopf and Zsigmondy, whereby the preparation is brightly illuminated with rays of light, falling in a direction at right angles to the axis of the microscope. The illumination is so arranged that a single layer of the preparation is lit up whilst the remainder is in shadow. With the help of this apparatus particles can be distinguished of a size of four-millionths of a millimetre. (The symbol  $\mu$  is used for  $\frac{1}{1,000}$  mm. and  $\mu\mu$  for  $\frac{1}{1,000,000}$  mm.) The effect of such an illumination may be realised by recalling the well-known appearance of particles of dust floating in a beam of sunlight penetrating into a darkened room, when the particles are viewed in a direction approximately at right angles to the beam of light.

A micro-chemical examination is of value for ascertaining the composition of the individual parts of a micro-organism. After treatment of the cells with reagents, the reaction brought about, and especially the colour reaction, may be studied under the microscope, with a view of determining the chemical composition of the part in question. As an example the proof may be cited that yeast cells, in the later stages of development, contain the reserve food stuff, glycogen (a carbo-hydrate). In order to detect this substance in the cell an iodine solution is added to the preparation (2 grammes iodine, 6 grammes potassium iodide, and 120 c.c. of water). The albuminoid

portion of the cell contents is thereby coloured bright yellow, whilst the glycogen assumes a reddish-brown colour. A further proof that the coloured part of the cell contents really consists of glycogen is obtained by warming the preparation to 70° C., whereby the colour reaction of the glycogen disappears ; on cooling down the colour reappears. If the cells are cautiously pressed under the cover-glass, so that they burst, it may be observed that the brown coloured glycogen is liberated as a fluid mass, which quickly dissolves in the surrounding liquid.

**Oil or fat globules** are often found amongst the granules that occur in the fully developed yeast cells. By the addition of 1 per cent. osmic acid they are coloured brown ; sometimes they assume a vermillion colour with alcanna tincture, and frequently they may be dissolved by treatment with alcohol-ether, benzene, or carbon bisulphide. The treatment must, however, be continued for some time ; the solution takes place more rapidly if the cells are crushed and the solvent brought into direct contact with the globules of oil. The **albuminoids** of the cells, as already stated, are coloured yellow, or yellowish-brown, with iodine ; with nitric acid and ammonia they are coloured lemon-yellow, and with Millon's reagent (mercuric nitrate), brick-red.

In the examination of fermented liquids micro-chemical methods are used to determine the character of any suspended matter other than micro-organisms. To clearly recognise the reaction of the tiny particles floating in the liquid, it is sometimes necessary to separate them by centrifugal action, and further to wash them free from the liquid. The **starch** or **paste cloudiness** which occurs in beer is produced by fine particles of starch or dextrin, which separate out when the liquor contains a given quantity of alcohol. They may be recognised by the addition of iodine, whereby they are coloured blue or brownish-red (dextrin). A **cloudiness** due to **albuminoids** is often produced ; it is due to flocculent, membranous, and often very irregular agglomerates, and to minute granules, which are either isolated or enclosed in these secretions. Such a formation frequently occurs in pasteurised beer ; it may be distinguished under the microscope by the readiness with

which it absorbs the colouring matter from iodine or from aniline dyes; it is coloured brick-red with Millon's reagent, and rose-red with Raspail's reagent (sugar and sulphuric acid). These bodies may also acquire a yellow colour (through the formation of xanthoproteic acid) by treatment with nitric acid and then with ammonia, or sometimes with the acid alone. **Glutin** cloudiness is a frequent form of albuminoid precipitation; it takes the form of a fine network throughout the liquid. At 30° to 40° C. the liquid becomes absolutely clear; on cooling, it again becomes cloudy. Under the microscope, fine flecks and small yellowish granules can be seen. According to Will, the latter consist of envelopes, more durable than the contents, which are easily attacked by water or dilute alcohol, acetic acid, or hydrochloric acid, and thus the sheaths become recognisable; in 5 per cent. potash, the whole granule dissolves. On warming, the contents, but not the sheath, are dissolved; hence the warm liquid is not always absolutely clear. Occasionally a cloudiness due to **hop resin** occurs in beer; the yellowish-brown globules are recognised by the vermillion coloration given by an alcoholic alcanna solution. A cloudiness of beer due to a **resin** derived from pitch has been described by Will; precipitations of this kind are coloured violet by a mixture of acetic anhydride and concentrated sulphuric acid. This reaction is specially marked when the granules are separated from the liquid.

## 2. Biological Research by means of the Microscope; **Moist Chambers.**

The examination described in the previous section can give but a limited insight into the nature of micro-organisms. A more complete knowledge of their life characters can only be reached through a biological and physiological investigation. The methods adopted have gradually reached a high stage of development, and **micro-biology** now stands as an independent branch of natural science, which has given results valuable both to science and to industry.

The subject of micro-biological research may be either a

**growth or an individual, a single cell.** In the first case, the certainty of the result is determined by the purity of the growth, and whilst the work is in progress this must be secured by the adoption of special precautions to be further described. In the second case, with which we now have to deal, the entire examination must be carried out under the microscope, special means being required to enable us to observe the series of changes that arise from the development and growth of the single cell. With this aim in view, Ranzier's moist chamber may be used (Fig. 3). This apparatus is made by grinding a slight hollow in the middle of a common object-glass; round this hollow a groove is made of greater depth to carry water. The drop of nutritive solution, which must be very small, is

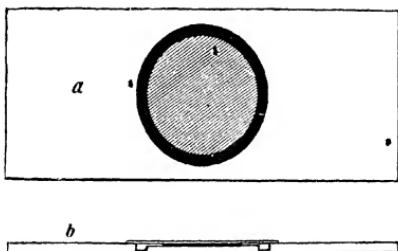


Fig. 3.

placed in the middle of the hollow and covered with a cover-glass, which extends beyond the groove. When the cover-glass is in place, it is cemented by means of vaseline, and the drop is thus enclosed between the cover-glass and the hollow of the object-glass, whilst the water in the groove prevents evaporation.

If by suitable dilution, care has been taken that only one cell has been sown in the drop of water, the study of its development may be extended for any length of time, with the certainty that all forms that appear are derived from one and the same individual. It is obviously a condition of this and all similar investigations that the liquid and the closed part of the apparatus must be sterile.

This chamber may be used again to decide whether fine

particles floating in a liquid are secretions or bacteria. Substances are added to the liquid which favour the growth of bacteria, and by lengthened observation of the behaviour of the particles it may be determined whether they propagate or not.

Amongst various kinds of moist chambers, that of Böttcher (Fig. 4), which now finds extensive use, may be mentioned. It consists of a glass ring fastened to a common object glass, and upon this a cover-glass is cemented with vaseline. The cover-glass carries on its under side a freely suspended drop containing the object to be examined. A few drops of water are placed on the floor of the chamber to prevent evaporation of the suspended drop. If the cover-glass has been completely freed from grease by cleaning with acid and ether, the drop will spread out to a thin layer, so that it may be observed under a strong power, and with a short focal distance. As the drop hangs freely, it is possible to lift the cover-glass

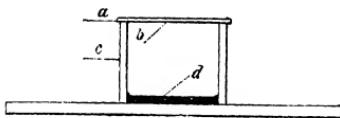


Fig. 4.

without disturbing the growth, if a sample is to be removed. If the cells are to be fixed, a little gelatine may be mixed with the liquid before sterilisation, as suggested by Brefeld. In his detailed researches on micro-organisms, Brefeld used this and other kinds of moist chambers, which are illustrated and described in Vol. IV. of his *Botanischen Untersuchungen über Schimmelpilze* (Leipsic, 1881). In order to secure the presence of a single cell in the drop, he diluted the infected liquid until this proved to be the case. If the organism demands a full supply of air to reach complete development, a stream of moist air may be passed through two tubes fixed in the sides of the glass ring.

By the help of such apparatus definite conclusions can be arrived at regarding the nature of the growth of micro-organisms, and this knowledge is essential for accurate work with mass cultures. To prepare the latter, the small pure

culture is transferred with every precaution from the moist chamber to a flask containing sterile liquid (see detailed description in a later section).

### 3. Sterilisation.

The principles of the whole technology of sterilisation, as well as the different kinds of apparatus required, were described in the early memoirs on spontaneous generation.

The details of the development of this subject in its historical setting are given with the description of the theories of fermentation in Chap. v.

Sterilisation of objects, whether a liquid or a piece of apparatus, means the riddance therefrom of all germs capable of development. This may be carried out either by removing all germs by mechanical means or by killing them by heat, or by the use of antiseptics. The choice of treatment is determined by the composition of the object to be sterilised, and obviously those means will be chosen that will render the adhering germs harmless, whilst producing the minimum of change in other directions. Sometimes, with this in view, the sterilisation can only be partial; for instance, if the properties of the liquid are changed to a great extent, by heating to the temperature at which the germs present would be killed, the lowest temperature must be found by experiment at which the organisms are so greatly enfeebled that they are no longer able either to develop or to affect the liquid. This is the object of pasteurisation, which will be discussed later on.

One circumstance which often presents great difficulties to complete sterilisation is this—that the great majority of micro-organisms occur in two different forms of growth, vegetative cells and spores. Whilst the former are usually killed at a temperature below the boiling point, the latter, and especially the spores of bacteria, can withstand prolonged heating at the boiling point; thus Flügge has isolated a species of bacteria from milk, the spores of which withstood boiling for four hours. When such spores are encountered, it is necessary either to boil for several hours, or to adopt a

considerably higher temperature; the latter alternative is specially necessary when dry heat is used.

(a) **Sterilisation of Glass and Metal Objects.**—Sterilisation, properly so called, must always be preceded by a thorough mechanical, and often by a chemical, cleansing. Articles of daily use in the laboratory, such as spatulas, needles, wires, etc., are heated directly in a flame, and allowed to cool in a space free from germs. Heavy pieces of apparatus, however, do not admit of this treatment; harm may be done by overheating while ensuring that every part of the object has been sufficiently heated, or the number of objects to be sterilised may be so great that it would take too much time to treat each singly. The apparatus must in this case be placed in special **sterilisation ovens**, where it is exposed for some time to a temperature at which it is believed that all germs will be destroyed.

**Dry or moist heat** may be used according to the nature of the article. Dry heat is a much weaker disinfectant than moist heat at the same temperature. To make certain that all germs are killed when using a hot air steriliser, the air must be raised to a temperature of  $150^{\circ}$  to  $160^{\circ}$  C., and the articles must be subjected to this heat for one to two hours. Some objects are wrapped in paper, others (*e.g.*, flasks) are closed with a cotton wool plug, which should be covered over with filter paper. If moist heat is required, the object can either be boiled in a water bath, or, better still, subjected to the action of steam. It is obviously of importance to see that the air is completely driven out, so that it cannot form a protecting layer, and prevent the steam from coming into contact with the object.

Either a current of steam may be used, or steam under pressure. In the first case, the apparatus is placed in a vessel provided with a perforated false bottom, with a sufficient quantity of vigorously boiling water below it. The steam escapes slowly, as the lid of the vessel is not air-tight, and the apparatus is gradually raised to the boiling point. By boiling in steam at  $100^{\circ}$  C. all vegetative forms are probably killed, together with many spores of bacteria and other resistant forms if the treatment is continued for an hour.

There are, however, spores which withstand this treatment ; thus, Sames and Christen have shown that the spores of certain species of potato bacilli which frequently occur in soil will withstand 10 to 16 hours' boiling. If, therefore, we have to do with material which has been in contact with the earth, higher temperatures must be employed, for it has been shown that the disinfecting power of steam rapidly increases when its temperature rises above 100° C. Steam under pressure is, therefore, used in a Papin's digester or autoclave, constructed to stand a pressure of several atmospheres. This apparatus is specially useful for the sterilisation of several liquids used in the laboratory. If small quantities of liquids are to be sterilised, a pressure of one atmosphere, corresponding with a temperature of 120° C., is sufficient, if applied for half an hour. During the cooling of any variety of sterilising apparatus, care must be taken that the incoming air is sterile, and this is secured by passing it through sterilised cotton wool.

(b) **Sterilisation of Liquids and Solid Nutritive Substrata.**—All germs can be removed from nutritive liquids by filtration, but this method of treatment, which is more troublesome than heating, is only used for liquids when their composition is affected by heat. Even in this respect it must be noted that filtration is not without effect, for the investigations of Flügge, Arloing, and others have shown that a filter retains or reacts upon certain of the soluble constituents, for instance, upon certain enzymes. As the filtering medium, either burnt clay, plastic charcoal, gypsum, asbestos, or kieselguhr may be used. The pores of these substances are very fine, and a thick layer must be used to ensure that even the smallest bacteria are retained. The pores are soon stopped up, and the filtration must then be hastened by pressure or suction. In laboratories, and for the filtration of small quantities of water, the Pasteur-Chamberland filter, consisting of burnt porcelain clay, and Nordtmeyer's filter made of compressed diatomaceous earth are frequently employed. These filters take the form of a hollow candle, closed at one end ; the liquid flows into the hollow and out by a tube fixed at the other end. To test such a filter, it is immersed in water, and air is blown into the cavity. If bubbles of air rise through the water, the filter is

evidently perforated, and is, therefore, useless. The first runnings of a filter, even a perfect filter, are not always sterile, and after a filter has been in use for a short time germs always pass through it. This happens because the germs at length grow through the pores, since it is almost unavoidable that substances which supply nutriment to the bacteria should not penetrate into the filtering medium. The surface of the filter must, therefore, be frequently cleaned, and the filter sterilised, which is most easily done by boiling it in water.

In breweries, the filtration of beer has been resorted to during the last few years, the filtering media commonly used being paper, cellulose, asbestos, etc. By such filtration brewers sometimes succeed, it is true, in freeing a beer of sound origin from deposits of various kinds, and in rendering it bright ; but, on the other hand, it has been directly proved by Thausing, Wichmann, Reinke, Lafar, and others that an indiscriminate employment of this method may prove extremely dangerous. If the filters are not effective, it may happen that only yeast cells are retained and not bacteria, which can then react with much greater energy upon the liquid. Another great danger lies in the fact that a filter, when it is imperfectly cleansed, may harbour colonies of different kinds of germs, causing the contamination of all beer passing through it. If a single cask in a cellar has become infected, and the filter is not effectually sterilised after the filtration of its contents, the disease will be communicated to the whole of the beer. It is, of course, a great mistake to use a filter which has been allowed to stand for a day without previous sterilisation ; the different species will have rapidly propagated in the favourable substratum, and will be swept off by the following filtration. In this stage of development the cells of wild yeasts are much more vigorous than those of the cultured yeasts, so that the disease organisms will multiply rapidly, and cause serious infection. A warning must be given against treating the filter with water at a temperature below the boiling point ; a thorough cleansing can only take place by prolonged boiling. By careful handling of the excellent filters now manufactured by several makers a more stable product can be obtained than that before filtration, as the experience

of the author has shown. It is certainly not allowable to lay it down as a general rule that beer must always be spoilt by filtration.

The filtration of milk of any biological importance has proved, so far, impossible, as a filter with pores large enough to allow the fat globules to pass will not retain bacteria, of which the vast majority are smaller than the globules. The filter is, therefore, only of use in removing the greater part of the dirt particles from the milk, and the micro-organisms that are attached to them. On a large scale sand and gravel filters are used. For instance, in the Danish system, as constructed by Busck, a vertical cylinder is used with perforated diaphragms, between which are packed layers of sand, the grains of which are coarser at the bottom and finer at the top. The milk is run in from below. In Kröhnke's construction the milk is passed through a cylinder partially filled with gravel, and carrying vertical diaphragms; the cylinder is rotated round a horizontal axis. On a small scale, the fresh warm milk may be filtered through cotton wool, a layer of which is inserted between two sieves; the filter requires renewing daily. A more complete biological purification has been attempted by pasteurisation.

The filtration of air is intended, not only to remove living germs, but also to remove all floating particles. It has already been stated that Schröder and Dusch accomplished this by means of a tube filled with closely packed cotton wool, and this still proves to be one of the best materials. In the laboratory such filters are used to seal test tubes and flasks. When they take the form of glass tubes, as in the Freudenberg flasks, it is unnecessary to protect the surface at the open end of the tube, but it is otherwise in the case of test tubes, where a great part of the filter is exposed to the dust of the air. Germs may easily grow on the cotton wool when it absorbs moisture. Flaming the surface is not always sufficient, and in such a case it is desirable to keep the tubes in an atmosphere free from germs. By the diffusion of air, which goes on through such small filters, evaporation takes place, and as a consequence the liquid becomes more concentrated, or the gelatine hardens on the surface. Such evaporation can be

avoided by the use of the flask constructed by the author (Figs. 8 and 9). On the large scale in breweries, yeast factories, etc., cotton wool packed in suitable vessels is also used for filtering, air, or else the air is led through a large number of layers of cotton wadding (Möller's filter). The complete sterilisation of the air on the large scale cannot always be attempted, and could not always be justified from an economic standpoint.

In the filtration of water on the large scale, the conditions existing in nature are imitated, where water is allowed to sink through successive layers of soil, and the organic residues and micro-organisms are deposited on the finer layers, until, at a given depth, the water is sterile. Artificial filters constructed of a number of layers of varying coarseness were first applied in London, and are now used in every country. Such a filter consists of a bed of large stones, covered with several layers of flints successively reduced in size so that the topmost layer is about the size of a pea, and on this is laid a layer of sharp sand about 5 feet in thickness, which has previously been washed. The water is first stored in a reservoir, where the larger particles settle out. When the filter is used for the first time water is led in slowly from below, so that all the air is driven out of the filter. It is then allowed to stand quietly for some hours before the true filtration begins. This must be carried out slowly at first, and then more rapidly. It has been shown that the distance between the separate particles of sand is greater than the bacteria, and, therefore, the retention of the bacteria is not due to the sand filter. While the water is standing quietly over the filter, slimy matters in suspension settle down and form a fine skin of slime on its surface. This retains a few bacteria, and as it always contains organic residues, it supplies nutriment for the bacteria, and as a consequence they multiply. A few bacteria settle on the grains of sand in the upper part of the layer, and these become slimy, and so arrest the bacteria subsequently carried down with the current of water. In this way the upper part of the layer of sand gradually fills up, so that the pores between the slimy grains of sand are now smaller than the bacteria, and then, for the first time, it can act as a true filter. It is now "ripe," and the water in the

lower layers of sand will be found to contain few bacteria. Thus it is the matter contained in the water itself that converts the upper part of the bed of sand into a filter. The necessary condition for satisfactory working is that the water shall flow slowly, in order that the bacteria and other particles may have time to settle on the slimy grains of sand, and also to prevent the skin breaking or the formation of channels through the bed of sand. The rate of flow must depend on the nature of the supply. If rich in bacteria, it should not sink more rapidly than 2 to 3 inches per hour. For the same reason the water level must be retained within certain limits. During the slow passage of water, the bacteria embedded in the upper part of the sand are able to retain some of the dissolved organic matter in the water, so that when it leaves the filter it should be free from fermentative and putrefactive components. A stage is reached at last when the pores are so completely filled with bacteria that the capacity of the filter is greatly reduced, and it is then renewed by removing the top inch of sand. This process can be repeated until a layer of sand 16 to 20 inches thick is left; the layer must then be restored to its original thickness.

As many different factors condition the activity of the filter, such as the changing biological contents of the water, alterations in temperature, etc., its activity must undergo many variations from a biological point of view, and this necessitates a continuous bacteriological control. Completely sterilised water cannot, of course, be obtained from such a filter, but it has been shown that in a good filter only one out of every 1,000 or more germs is transmitted.

Considerable use is also made of "rapid" filters. They may be constructed of sand, wood charcoal, etc., so as to allow of the passage of large quantities of water. The filtration is combined with the use of chemical precipitants, whereby the greater part of the slimy particles and organisms are separated from the water.

\* The treatment of water with ozone is dealt with in a subsequent section.

The exact method of sterilisation of liquid and solid substrata by means of heat is determined by their chemical and

biological nature. The methods employed include the use of a current of steam, steam under pressure, boiling in water or on the sand bath, and the treatment may be prolonged for a considerable period if it is desired to kill not only vegetative cells, but also spores. In either case it is obviously of importance to take care that during the subsequent cooling only sterile air is admitted to the vessel. This is secured in the case of Pasteur flasks by the use of a tube with two bends, in which any germs that are sucked in are deposited; in the case of Erlenmeyer and Freudenreich flasks, by sealing them with cotton wool filters. Whilst the hopped wort commonly used in zymophysiological laboratories will stand boiling on the sand bath, and after a comparatively short boiling can be preserved unchanged, wort gelatine and other gelatines cannot stand treatment on a sand bath or such prolonged boiling on a water bath or in steam, that will ensure the destruction of all spores, because there is always a danger that after such treatment the gelatine will no longer solidify at a temperature of 25° C. The same difficulty is met with in the sterilisation of the mash in distilleries and of wort in the air yeast factories, owing to the great separation of albuminoid substances which takes place at the boiling point, causing a complete change in the character of the liquid. For this reason it is impossible to apply all the results of experiments obtained with properly sterilised liquids to the very different circumstances that obtain in practice. In all such cases use is made of the method of fractional or discontinuous sterilisation introduced by Tyndall. Its object is to bring about the germination of spores of bacteria and similar resistant organisms by maintaining the material at a gentle heat for some time, so that the cells may subsequently be killed at comparatively low temperatures. The material is first warmed, perhaps to a temperature of 70° C., or it may be heated for a quarter of an hour in a current of steam in order to kill the vegetative cells. It is then maintained at room temperature, or, better still, at the most favourable temperature for the development of spores (about 35° C.), and after the lapse of a day, or even of a shorter period, when it is assumed that germination is complete, the material is again heated. By repeated treat-

ment of this kind it is possible to eliminate all spores and to kill all vegetative cells. This obviously depends, however, upon the regular germination of the spores. The treatment does not absolutely guarantee sterility, and before either liquids or gelatines are used they must be kept under observation for a considerable time. In many cases filtration is to be preferred to discontinuous sterilisation. The liquids in daily use that are prepared with the help of micro-organisms, beer, wine, vinegar, etc., always contain a residue of these micro-organisms in a more or less vigorous condition. It is desirable, by heating them, to arrest the fermentation. The safest course is to sterilise the liquids, but as the temperatures required to effect sterilisation usually produce great changes in the liquids, it is necessary to limit the treatment to a temperature that will suffice to weaken the micro-organisms, so that under normal conditions they are extremely unlikely to propagate or to bring about further fermentation ("pasteurisation"). It is difficult to determine the best method when the nature of the liquid will not admit of a high temperature being used, while the result must depend upon the character and the activity of the different micro-organisms present, as well as upon the chemical composition of the liquid. It is, therefore, impossible to establish any general rule. It is essential in each case to determine experimentally both the temperature and the time of treatment, after forming a judgment as to what micro-organisms are present in the liquid. In the case of beer different temperatures are used—heating from 50° to 60° C. for two hours, or from 65° to 70° C. for half an hour or more—and for wine, two hours' heating at 45° to 50° C. (*C. Schulze*). A slow cooling down after pasteurisation has often been experimentally proved to give better results than rapid cooling. The determination of the right temperature is obviously rendered more difficult if the liquid harbours different species of yeast, and still more so if at the same time the development of bacteria has taken place, especially those species that form spores. It has been proved that when the heating exceeds certain limits, the flavour either of beer or wine quickly deteriorates, which is probably due in the first place to the decomposition of albuminoids.

If the liquid is particularly sensitive to high temperatures, it is necessary to fall back on the method of discontinuous treatment, whereby the liquid is heated to a moderate degree several times, with a suitable interval between each heating. Frequently the alteration in taste produced by pasteurisation can be partially removed by subjecting the liquid for a time to a low temperature. A special difficulty met with, particularly in the case of beer, is that during storage or transport, particularly at low temperatures, the pasteurised liquid develops a turbidity, or forms a deposit, consisting usually of albuminoid substances separating in the form of small granules, or, in difficult cases, in flakes and skin formation. It has usually proved necessary to control the preparation of the malt if such a calamity is to be avoided. Care must be taken that a slow and sufficiently advanced development of the grain has taken place, accompanied by a full transformation of its contents. Further, it is obvious that the fermentation should have been vigorously carried out, and in this connection it is particularly necessary to adopt pure ferments. By cooling the beer to a low temperature before filtration and pasteurisation it is possible to avoid the subsequent separation, as part of the material in question is separated in the cooling process.

In dealing with milk, heat is applied in the same way. In this case the greatest possible difficulties are met with owing to the great range of micro-organisms present in milk (lactic acid bacteria, putrefactive bacteria, hay and potato bacilli, etc.), many of which are only killed at a high temperature, owing to their power of forming spores. Heating the milk further results in separating or modifying components, which may be of extreme value in nutrition (*e.g.*, the enzymes), even at comparatively low temperatures. Pasteurisation at temperatures considerably below the boiling point may result in the milk bacteria being killed, whilst the putrefactive bacteria remain alive. As a consequence, the latter, freed from competition, multiply rapidly, and form putrefying matter in the milk, and this may occur to a considerable extent if the milk is not stored at a very low temperature. Actual sterilisation can only be secured if the milk is heated

for an hour or more at a pressure of half an atmosphere, corresponding to 112° C. If the object is simply to destroy the pathogenic organisms that are present, especially the tubercular bacilli, it is only necessary, according to Bang and Weigmann, to heat for a few minutes at 85° C., or for a period of from a quarter of an hour to one hour at 65° C. The problem how to secure a product free from any organisms capable of development, and yet of good nutritive value, has not yet been solved, although long-continued treatment at 60° C. has proved of some value. (Absolute certainty cannot, of course, be obtained in this way.) The tendency is, however, to establish a stringent control of the milch cows and of the milking operations, in order to make sure of a healthy product.

Sterilisation of air can be best secured, as already stated by means of cotton wool filters. Sulphuric acid or brine baths, cloth filters, etc., are less effective. In the laboratory, where it is often necessary to carry out work in sterile air, glass cupboards are used, the front of which can be sufficiently raised to admit the arms. Some time before using the cupboard the whole of the inner surface is washed over with either mercuric chloride solution or 60 per cent. alcohol, and the cupboard is then closed. Any particles and germs floating in the air will sink to the moist floor, and will be retained there.

In breweries and other branches of the fermentation industry, the fermentable liquid is sterile at a particular stage in the manufacture, at the moment when the boiling is completed. After the zymotechnical analysis of air had shown that it may convey disease germs to the fermenting liquid, attempts were made to protect the wort during the cooling operation against such infection by the use of closed cooling and aërating apparatus, closed fermenting vats, and storage casks, and by the sterilisation of the incoming air through cotton wool filters. These precautions, together with the use of an absolutely pure yeast, should, theoretically, produce an absolutely pure product. Incidentally, one important practical object was secured, for by blowing in a powerful stream of air during the fermentation, and by the removal of carbon dioxide, the rate of fermenting was greatly increased, and an earlier clearing of the liquor took place. The difficult

problem is to maintain such large vessels in a condition of absolute cleanliness.

The experience of many years has, however, shown that in breweries with open refrigerators and cooling apparatus, open fermenting vats, and ordinary storage casks, a product can be obtained with such a small content of harmful germs that they have no practical influence on its quality, notwithstanding the fact that the wort, especially on the refrigerators, is exposed to a number of foreign germs. It has now been proved that the harmfulness of the atmospheric germs in the fermentation industry has been greatly exaggerated, for in competition with the enormous number of yeast cells which are established in the wort, the vast majority of these germs never come to development. If it happens that, notwithstanding the use of pure yeast, the product is strongly contaminated with disease organisms, the explanation is, in the great majority of cases, that these are developed in the plant itself. It is from the surface of the different vessels employed that the dangerous carriers of disease have developed, just because a rational method of cleansing has not been adopted. The chief importance must be attached to those stages in the process where the liquid is longest under treatment, in the fermenting vats and storage casks. In order to purify these vessels, as well as the connections, disinfectants are almost always used, and it may be remarked that a summary treatment with these is not sufficient. This, at any rate, holds good for wooden vats, in which it has often been proved that notwithstanding disinfection the disease germs retain their hold. A special investigation must, therefore, be made into the physical character of the vessels, and the necessary precautions must be adopted. If in this way a rational method is worked out, it will be found that the atmospheric germs exercise no noticeable influence on the course of fermentation or on the character of the product, since no opportunity is given for them to establish themselves in the plant.

Under special circumstances chemical reagents are used for disinfection, the **antiseptics**. The ground work of the technical application of antiseptics was laid by Schwann, who proved in 1839 that yeast cells die under the influence of certain

chemicals. More recently the knowledge of antiseptics has been greatly extended by R. Koch. As in the case of the action of heat, so the individual species react differently towards the various antiseptics. Moreover, one and the same species of vegetation may react differently towards the same reagent, and that not only because the spores possess a greater power of resistance than the vegetative cells, but also because the activity of the latter plays a part. One practical problem is to determine how far the antiseptic can be diluted without ceasing to react. Whilst with a given concentration the antiseptic may prohibit life, with a greater dilution the action only restricts development, and with still greater dilution, if any further influence is felt, it may take the form of stimulating both the development and activity of the organism. Many organisms possess a special power of accommodating themselves to strong doses of antiseptics.

Disinfectants are placed on the market either in a solid or in a concentrated liquid condition. Their antiseptic power must first be determined by experimenting with the groups of micro-organisms which may be encountered in the fermentation industry. Once the limit of their activity is determined, it is necessary to ascertain how rapidly a given dose operates. Should it prove that the action is too slow for practical application, other degrees of dilution must be tested until the minimum dose is found which will kill the micro-organisms in a short time (*e.g.*, in thirty minutes).

Flasks of 15 c.c. capacity, provided with ground glass stoppers, are used for the test. These are filled almost to the top with the disinfectant, and after a pure culture of each species has been placed in the flasks, they are thoroughly shaken.

When the action is completed, every trace of the reagent must be removed from the vegetation by washing, and a sample of the growth is transferred to a suitable nutritive substratum, and exposed under the most favourable conditions. It must be maintained at a constant temperature, which should be higher than that of the room. Liquids are to be preferred to gelatine, because the nutritive value of the latter is generally smaller. Finally, the observation of such growths must

extend over a considerable period, as it often proves to be the case that the cells have not been killed, and after a considerable time they may germinate again. The degree of dilution at which an antiseptic operates restrictively on species is usually dependent on whether the action takes place in a nutritive fluid or not. In the first case, the chemical nature of the liquid obviously has considerable influence. Thus, for instance, liquids which are rich in albuminoids weaken the effect of many poisons. In a determination of this character for industrial purposes it is usual to limit the solvent to some particular fluid.

As an example of the part that the solvent plays, the classical work of Koch in 1881 may be mentioned, which led to the proof that many antiseptics may wholly or partly lose their power according to whether they are dissolved in water, in ethyl or methyl alcohol, ether, or acetone. In this connection an important fact may be noted. The addition of sodium chloride to certain antiseptics (*e.g.*, to carbolic acid or mercuric nitrate solution) causes an extraordinary increase in their antiseptic power.

Temperature, also, has an influence on their action ; the higher the temperature, the greater their activity. On the other hand, a dilute antiseptic exhibits the least restrictive power at that temperature which is most favourable to the organism.

Numerous investigations regarding the influence of antiseptics on different species of micro-organisms have shown that no general rule can be traced. One species may be much more resistant to one poison than many other species, whereas it may exhibit little resistance to another poison. The destructiveness of a given substance cannot, therefore, be defined in general, but only its behaviour towards a particular species.

The application of antiseptics for the cleansing of vessels, etc., must always be preceded by a thorough mechanical cleaning.

Antiseptic substances are partly inorganic, partly organic. Amongst the mineral substances, the first place must be given to **mercuric chloride**, on account of its extremely poisonous character. It is used in the laboratory in a solution of 1 gramme per litre of water, but it is impossible to use it in

technical work. Like most of the other mercuric salts, mercuric chloride belongs to that class of bodies which produce insoluble compounds with albuminoids, and thus do not react completely with bacteria. Attempts have been made to overcome this difficulty in such cases by the addition of a small percentage of sodium chloride. **Hydrofluoric acid** and its salts also belong to the most powerful antiseptics, especially as regards bacteria. **Ammonium fluoride** is generally used, and has a wide application. **Chlorine** is used in the fermentation industry in the form of chloride of lime, but it is only applicable within certain narrow limits, owing to its strong and pungent odour. Another compound, sodium hypochlorite (**antiformin**), which has a weaker smell of chlorine, is more widely used. Chlorine is also used to disinfect water. For this purpose small quantities of chloride of lime are used, and after a short time the chlorine is fixed by the addition of calcium bisulphite. **Sulphurous acid** is used sometimes in the form of gas or of an aqueous solution, and sometimes as calcium bisulphite or sodium sulphite. It is used, not only as an antiseptic, but also as a means of restricting fermentative activity. This, as well as several of the above, usually appears to attack bacteria more strongly than yeasts in high dilutions. **Soda** is of value as a means of cleansing, as well as disinfecting, and this applies also to **lime**. Lastly, two gases must be mentioned which are now coming into use, **ozone** and **hydrogen peroxide**, the latter having an even greater disinfecting power than ozone.

Amongst organic antiseptics, **formaldehyde** has found very extended application during the last few years, on account of its great antiseptic power. Thus, the spores of many resistant bacteria are killed by the application of a 0.1 per cent. solution for an hour. On the other hand, this reagent, when used in the form of vapour, has little action on man and the higher animals. Its vapours appear principally to attack the surface of articles, as its power of penetration is not great.

A series of antiseptics which have proved of special importance in laboratory studies of fermentation includes **ether**, **chloroform**, and more especially **acetone**, **toluol**, and **thymol**, because they possess the valuable property that, while they

have the power of killing germs, they do not destroy enzymes. This fact has proved of importance in advancing recent studies of enzymes, etc., where it is necessary to inhibit the action of micro-organisms on the susceptible liquids employed. R. Koch first proved the antiseptic action of ether and alcohol, and recent research has brought to light the interesting fact that it is not absolute alcohol, but a 50 to 60 per cent. alcohol that exhibits the strongest disinfecting power. This may be explained by supposing that absolute alcohol absorbs moisture from the surface of the cells, and, therefore, makes them more resistant. The vapour of 75 per cent. alcohol appears to be equal in its action to a current of steam, and a still more powerful action is exhibited by a mixture of alcohol vapour of this strength with formaldehyde vapour. The mixture may be used to disinfect fabrics which would suffer by exposure to a temperature of  $100^{\circ}$  C.

Carbolic acid (phenol), which plays an important part in medicine as a powerful antiseptic, cannot be applied in the fermentation industry, owing to its penetrating odour, but owes its interest to the fact that it does not attack enzymes. On the other hand, a whole series of compounds, of which carbolic acid is a component, are made use of in practice.

The raw materials of the fermentation industry (rye, wheat, barley, etc.) contain peculiar compounds which, according to the researches of Jago, Delbrück, Lange, Henneberg, Hayduck, and Seyffert, act as poisons to yeast, and these are assumed to be of an albuminoid character. This action may be observed in the crushed grain or in an aqueous extract if the yeast is added in presence of sugar dissolved in distilled water.

In their reaction to such influences, the yeast species do not behave uniformly. Thus, under certain conditions a stimulus may be given to some species, whilst under other conditions the poisonous substance may act destructively even in minute doses. Such is the case with the poisonous body present in rye bran, and also with that contained in a dilute hydrochloric acid (0.1 per cent.) extract of wheaten flour, in their action upon brewer's yeast, whilst with the addition of calcium carbonate, soda, gypsum, etc., the action of these reagents on the yeast is prevented.

As stated above, it has been proved that very minute quantities of poisons may have such an influence on micro-organisms that they actually stimulate them to more rapid growth, often one-sided; it may be the development of the vegetative organs at the expense of the organs of propagation, or again by bringing about an increase of the fermentative activity. In a few cases that have been closely examined it has been proved that the minute doses which can produce such an action have fairly well-defined limits; the least excess brings about the opposite action—a weakening of the organisms in question. Thus a minute dose of a copper salt assists the development of the mould, *Aspergillus niger*, to a very great extent. In the same way Biernacki found that the addition of copper sulphate in the proportion of 1 : 600,000, added to the nutritive value of the liquid, and increased the activity of the cells. In larger quantities copper salts exercise a destructive action on yeast; care must, therefore, be taken that when pure cultures of yeast are introduced into copper vats, these should be carefully tinned. Hayduck (1881) found that small quantities of lactic acid (about 0.5 per cent.) favour the propagation of yeast, and that anything up to 1 per cent. of lactic acid, under the usual technical conditions, is favourable to the production of alcohol. Heinzelmann proved in 1882 that salicylic acid in the proportion of 0.1 gramme per litre reacts favourably on yeast cells, and H. Schulze (1888), that minute traces of poisons, such as mercuric chloride, iodine, chromic acid, and lactic acid, have the same action (e.g., mercuric chloride in a dilution of 1 : 500,000). Hirschfeld found that by the addition of 0.01 to 0.02 per cent. of hydrochloric acid the acetic fermentation is very considerably quickened. Richet proved a similar relation holds good with the lactic acid bacteria, while the addition of 0.5 mgm. of mercuric chloride, or of copper sulphate, per litre, intensifies their fermentative activity. In the same way Effront found that minute quantities of sulphuric acid and of fluorides have a stimulating action in nutritive liquids, both on the rate of propagation and the fermentative capacity of yeasts, but that this varies with the yeast species.

The fungi have a curious power of accommodating them-

selves to poisons. By long-continued cultivation it has proved possible to introduce large quantities into nutritive substrata, and at the same time it has been noted in several cases that a marked change of character takes place. It has, however, proved impossible to fix these newly acquired characters; they are of a purely transitory kind. As soon as the poison is removed the growth reverts to its original character. From the numerous examples, we select the following:—Galeotti accustomed *Bacterium prodigiosum* to grow on a 2 per cent. carbolic acid nutritive gelatine. Pulst accustomed *Penicillium glaucum* to withstand continually increasing quantities of poisonous copper salts, whilst its conidia germinated more rapidly than usual.

The results obtained by accustoming yeasts to the presence of certain poisons are of special interest in the technology of fermentation. For example, the yeast in distilleries may work in a mash which by treatment with a disinfectant has been rendered more resistant to bacteria, a process which takes the place of the usual souring with lactic acid. For this object sulphuric acid, hydrochloric acid, and hydrofluoric acid have been made use of. Effront proved that much smaller quantities of hydrofluoric acid were required than of the other two. In consideration of the different extent to which the yeasts are attacked by the hydrofluoric acid (or fluorides), Effront tried by special cultivation of yeasts to accustom them to work in a mash which contained so much of the reagent that the bacteria were suppressed. He found that the addition of 300 mgm. of hydrofluoric acid to 100 c.c. of liquid completely inhibited the growth of yeast, whilst its fermentative activity was only restricted. If, however, the yeast is gradually accustomed to the poison, beginning, for example, with 20 mgm. per 100 c.c., and rising by degrees to greater doses, a race of yeast will be formed that can multiply even in presence of the original dose. In presence of 200 mgm. per 100 c.c. the fermentative power of the yeast is increased, according to Effront, if it is introduced into a mash which also contains fluorides. In practice about 10 grammes of hydrofluoric acid are used for every hectolitre of mash. Even if this process succeeds in suppressing bacteria in the mash, which is not always the case,

other difficulties may arise when wild yeast are present, for these, according to Holm and Jörgensen, are stimulated in their development by the presence of hydrofluoric acid in the mash.

#### 4. Disinfection in Practice.

It has become clear, within the last few years, that the harmfulness of the germs in air and water has been greatly exaggerated, and that far and away the most important source of danger is to be sought in the growth of foreign organisms in the plant itself. The natural result is that increasing attention is being paid to a rational scheme for disinfecting the plant. As the raw stuffs used in breweries, distilleries, etc., form an admirable nutritive medium for micro-organisms, the distribution of these throughout the plant is much more widespread than usual, and it is often necessary, in addition to mechanical cleansing, to attack them by direct antiseptic means. By determination, on the one hand, of the maximum limit of such poisonous substances that can be allowed, and, on the other hand, of the necessary means to secure the desired object, the practical conditions are established. The concentration must not exceed what is absolutely necessary. In the use of antiseptics it is essential to follow a recognised plan. A summary disinfection is insufficient if the individual parts present different possibilities for the development of foreign organisms. It is, therefore, necessary from time to time, and that frequently, to overhaul every single point in practice, before being able to say exactly where a particular infection has appeared. At certain points antiseptics must be discarded and mechanical means adopted. This is the case when the infection has penetrated so far into the material that the disinfectant is no longer able to attack it. This may occur in the great majority of wooden vessels as they are usually prepared.

As many micro-organisms form slime, and may produce thick deposits when allowed an undisturbed development, it is often necessary to use a solvent of the slime before proceeding to actual disinfection, if the germicidal substance is not capable of completely dissolving the slime.

It is an established rule that two disinfectants should not be used simultaneously, or one immediately after the other, especially if their composition is unknown ; otherwise there is danger that they may neutralise each other's action. Thus, chloride of lime and calcium bisulphite should never be used at the same place.

The literature of antiseptics used in the industry—Will, Lindner, Brand, Schönfeld, etc.—has grown to considerable proportions. A short *résumé* of the methods of application of the respective substances follows.

**Ammonium fluoride**, especially the acid salt, has a very wide application, owing to its great antiseptic power. It is a crystalline powder, readily soluble in water. In the pure condition it contains about 35 per cent. of hydrofluoric acid ; the commercial product, however, contains a less quantity, and sometimes not more than 20 per cent. It is used for the treatment of pipes, vats, etc. Pipes are filled with a solution containing about 0.5 per cent. In rinsing out vats a 3 to 5 per cent. solution must be used. Ammonium fluoride is not suitable for the treatment of metal, as it slowly attacks it. After treatment, a very thorough washing with water is necessary.

**Formalin** has also been very largely applied in practice. It is an aqueous solution of formaldehyde (40 per cent. by volume or 37.2 per cent. by weight), and it forms one of the most powerful antiseptics. As it does not attack metal, it can be applied more generally than the fluorides. It may be used in the form of gas by soaking cotton wool or cloths in formalin, and hanging them up in the area to be disinfected, or it may be applied in specially constructed lamps, in which, by the imperfect combustion of methyl alcohol, formaldehyde is produced. The most frequent and most successful method of application is, however, to dilute formalin with water, and apply it as a spray to the walls of vats, etc. A solution of 0.5 per cent. of formaldehyde (about  $1\frac{1}{2}$  litres of the commercial article to 100 litres of water) is most generally applicable. The vessel must then be well rinsed with water, and if the odour cannot be got rid of, ammonia may be applied.

**Chloride of lime** has been used for many years, on account

of its powerful disinfectant properties. Its strong odour limits its application. It is especially used to disinfect racks and slimy walls in rooms where fermentation is going on. To disinfect filter bags in breweries, which often harbour large colonies of bacteria and wild yeast, Will recommends an application of chloride of lime in a solution containing about 1 per cent. of active chlorine (3 to  $3\frac{1}{2}$  kilos. chloride of lime in 1 hectolitre of water). The mixture of water and chloride of lime is allowed to stand for some time, with occasional stirring; the clear liquid is then decanted and applied to the filter bags, which are afterwards repeatedly rinsed with water. The dangerous development of micro-organisms on the filter bags may be avoided by cooling down the beer to the lowest possible temperature during filtration.

**Antiformin** is a chlorine preparation which has found considerable application in recent times. It is a clear liquid with a strong alkaline reaction and a weak odour of chlorine. It consists of a crude sodium hypochlorite (*cf. Eau de Javelle*), and is prepared by decomposition of chloride of lime with soda. The solution is then separated from the precipitated chalk, and caustic soda is added. The liquor contains more than 4 per cent. of active chlorine, and not only possesses great antiseptic power, but also quickly softens organic substances such as sediment, wort, crust, yeast, and slime, so that they can easily be removed. In other words, it acts both as a cleansing and as a disinfecting agent. Care must be taken, however, in applying it to infected wood; for instance, to the staves of a fermenting vat, as the reagent, owing to its solvent power, can penetrate so far that it is difficult to remove it by rinsing with water. It may be applied in a dilution of 1 to 20.

**Antigermin** appears to be specially adapted for washing down walls. It consists mainly of a copper salt of an organic acid, and the aqueous solution is without smell. It should be dissolved in boiling water, and mixed with lime before applying.

**Montanin**, which is also free from smell, is equally applicable to walls and to connecting pipes, vats, etc., but the latter must always be well rinsed. It is a by-product of the glazed-

ware industry, and contains about 28 to 30 per cent. of hydro-fluosilicic acid (as aluminium fluosilicate) in a clear solution, pale green or yellow in colour, and feebly acid. The protection of walls by means of this preparation depends upon the pores being sealed by the formation of calcium fluoride, alumina, and silica, imparting to the wall a hard and smooth surface.

**Mikrosol** appears in commerce as an acid green paste containing about 10 per cent. of copper phenolsulphonate, and smaller amounts of copper sulphate, free sulphuric acid and hydrofluoric acid. It is applied to walls in the form of a 2 to 4 per cent. solution.

**Antinonnin** is largely used in order to coat moist walls, and is an excellent preventive of dry-rot, and protects woodwork from worms, etc. It forms a red viscous mass consisting of a potash compound of kresol mixed with glycerine, soap, etc. It is soluble to the extent of 5 per cent. in water. It does not attack either metals or organic substances, and, according to Aubry's investigations, may be applied to advantage throughout the brewery, where it cannot come in contact with beer.

**Pyritic**, a new preparation, is a white powder soluble in water to form a colourless and odourless solution. According to Wichmann, a 2 per cent. solution forms a very powerful disinfectant, which does not etch or attack either metal, wood, or glass. It may be applied anywhere, inside or outside. It can be kept for a long time without losing its activity.

**Sulphurous acid** is one of the oldest antiseptics, and is still frequently used for casks. A piece of linen which has been dipped in molten sulphur is set alight and introduced into the cask. The fumes do not, however, penetrate sufficiently to sterilise badly contaminated casks. Hops and occasionally malt are also treated with burning sulphur. In wine fermentation sulphurous acid is sometimes added to the must, to destroy the spontaneous germs before adding pure wine yeasts. **Calcium bisulphite** forms an energetic reagent, and usually contains about 7 per cent. of sulphurous acid. Diluted from three to six times with water, it forms an admirable agent for the treatment of vats and other apparatus, and is especially deadly to moulds.

**Salicylic acid** has also been applied to vats in the form of a dilute alcoholic solution, which is painted on to the surface, allowed to react for some time, and then washed off with an alkaline liquor, and finally with water.

Amongst weaker antiseptics, lime and soda may be mentioned. **Milk of lime**, freshly prepared, forms an excellent disinfectant for walls and ceilings, but as soon as the lime has absorbed carbon dioxide from the air it ceases to have any value. **Soda**, in the form of a 5 to 10 per cent. solution in warm water, is an excellent reagent for dissolving slime from connecting pipes, etc. It must, however, be very thoroughly removed by washing first with warm, and then with cold water. A very dilute soda solution (0.1 to 0.3 kilogramme per hectolitre of water) is of value in swilling new chips. Soda is not well adapted for disinfecting fermenting vats, as it imparts a rough surface to the wood.

One of the most important disinfectants throughout the fermenting plant is **steam**, if care be taken that every part of the vessel to be treated is exposed to its action. Connecting pipes may be sterilised by steam if they do not exceed a certain length.

In distilleries, **sulphuric acid** is used as a disinfectant in the mash to inhibit the growth of foreign bacteria, and to restrict that of yeast. Its application must, however, be kept within certain limits, as the yeast would otherwise be damaged. According to Hayduck, 0.024 per cent. can be used, and even 0.05 per cent. does not appear to prevent the development of yeast.

**Ozone** has found application, in particular for disinfecting water. In order to bring the gas into close contact with water, the latter is sprayed over a fine-grained material, where it comes in contact with a stream of ozone prepared by means of a high tension electric current, discharged from two electrodes of special construction. It has proved possible by this means to kill a very large proportion of the organisms in water (see the researches of Calmette, Schüder, and Proskauer, Ohlmüller, etc.).

**Hydrogen peroxide** has also been applied to disinfecting water, and preserving milk by Budde's process, which consists

in the application of 0.036 per cent., after which the milk is maintained for three hours at 50° to 52° C.

### 5. Flasks.

All vessels in which cultures are made must satisfy the condition that they are proof to every contamination from without. **Pasteur flasks** satisfy this demand in the highest degree. The illustration (Fig. 5) shows this flask in the improved form employed in the Carlsberg Physiological Laboratory. When the hopped wort (preferably filter-bag wort) is boiled, the steam first escapes through the straight tube, attached to which is a short piece of rubber tubing;



Fig. 5.—Pasteur's flask.



Fig. 6.—Chamberland flask.

when this is closed (after boiling for about half an hour) the only outlet for steam is through the bent tube. About twenty minutes later, the flask is removed from the sand-bath. During cooling the germs are deposited in the lowest part of the bent tube, or are not carried beyond the enlargement of the tube, and, therefore, do not come into contact with the liquid. Hence, it is evident that the lower part of the bent tube must be heated whenever the flask is to be agitated or emptied through the straight tube. If the flask is to be opened and connected with another flask, this must be effected either in a small sterile space, or else the opening and connecting must be carried out in a flame. A Bunsen burner is placed directly in front of the operator.

the flask to be emptied to the *left*, and the one that is to receive the liquid or culture to the *right*, alongside the burner. Then the tube of the left-hand flask is opened *in the flame* by quickly removing the rubber tube with its glass stopper ; while the open tube is in the flame, the glass stopper of the flask to the right is quickly withdrawn, and the hot tube of the first flask is connected with the rubber tube of the second flask after the tube has been cooled. The liquid is poured into the second flask, the bent tube of the first flask being at the same time heated. Then the side tube of the left flask is again introduced into the flame, while the stopper of the right flask is replaced directly after it has been passed through the flame ; finally,



Fig. 7.

the left flask is closed *in the flame* with its tube and stopper. When the operation is quickly performed the danger of contamination is almost excluded.

Pasteur flasks will be found indispensable in certain operations ; for instance, in physiological researches where large quantities of liquids are dealt with.

The **Chamberland flask** (Fig. 6) is closed with a ground cap, which terminates in a short, open tube ; this tube is filled with tightly-packed sterilised cotton-wool.

The **Freudenreich flask** is constructed on exactly the same principle (Fig. 7, centre) ; it has, however, a cylindrical shape.

These flasks must only be opened in a sterile cupboard. When gelatine is used the flask must be opened with the mouth downward.

For special purposes the **Hansen flask** (Fig. 7, left) is employed. The ground cap is provided with a cotton-wool filter, and the flask has a small side-tube closed with an asbestos stopper. This flask is used in the author's laboratory for the dispatch of small cultures or of samples from the propagating apparatus.\* For this purpose the lower part of the flask is filled with cotton-wool, and some cotton-wool is lightly packed into the cap. The asbestos stopper and the lower edge of the cap must be covered with sealing wax.

A flask (Fig. 7, right, and Fig. 8) constructed by the author



Fig. 8.

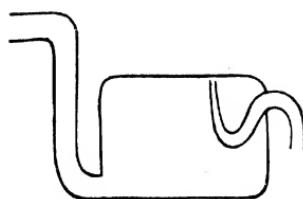


Fig. 9.

carries a small, bent, and open tube in the cap, as a prolongation of the cotton-wool filter. By this means it has proved possible to prevent the evaporation of the contents of the flask for several years, provided that the lower edge of the cap and also the lateral tube are well closed. This flask is used for preserving pure cultures of yeast in a 10 per cent. saccharose solution inoculated with a trace of the yeast. The flask is also suitable for a prolonged preservation of gelatines, if the surface is to be prevented from stiffening. Another flask constructed by the author, shown in section in Fig. 9, is also used for storing pure cultures of bottom and especially of top yeasts which will not stand vigorous shaking; when further

\* This apparatus is described in Chapter vi.

development is required a drop of the yeast is transferred to a Pasteur flask containing wort. The small bent open tube on the right has its outer extremity packed with cotton-wool, to filter incoming air. The wide tube on the left, which is closed with an asbestos stopper, has its lowest bend on a level with the bottom of the flask. If this tube is connected with the side tube of a Pasteur flask in the flame, and then suction applied to the bent tube of the flask, a minute part of the yeast lying on the bottom of the small flask will be sucked into the Pasteur flask, without disturbing the remainder of the yeast deposit. If it is necessary frequently to remove a small portion of a culture, this process may be recommended.

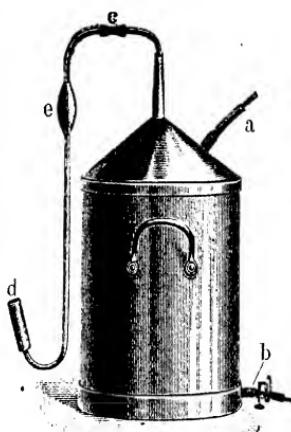
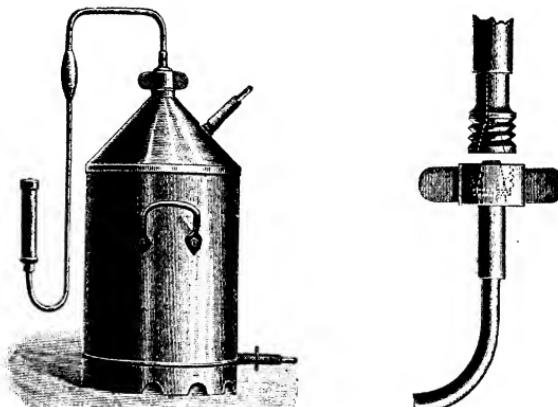


Fig. 10.—Carlsberg flask—Old model.

Fig. 11.—Carlsberg flask—New model. *b*, Connection between the flask and the bent tube.

For the development of very large cultures the Carlsberg vessels (Figs. 10 and 11) are employed. They have a capacity

of 10 litres, are made of tinned copper, are cylindrical in shape, and conical at the top; at the apex of the cone a twice-bent tube (*c d*) with or without an enlargement (*e*) is either soldered into or screwed into the flask. At one side of the cone is the inoculating tube and glass stopper (*a*), and near the bottom of the vessel is another tube (*b*) for drawing off the fermented liquid and the yeast. This tube is provided with a pinch-cock. When the liquid is sterilised, the bent tube is closed with an asbestos or cotton-wool filter, which is tightly packed on to the end (*d*).

In the new model (Fig. 11) the bent tube is ground into the upper part of the flask, and fastened by means of a screw, allowing the whole of this part to be detached, when the flask is to be cleaned; the filter is screwed into the end of the bent tube.

#### 6. Nutritive Substrata.

With regard to the nutritive substrata, the problem naturally consists in finding those which are best suited to the respective organisms. If they also possess the advantage of being less favourable for the development of competing forms, it is a great point gained. The fact must, of course, be borne in mind, when comparative investigations are made in different directions, that the nutritive liquid must always remain the same.

For the investigation of yeasts, hopped-beer wort forms the best nutrient. It is best taken from the filter-bags, because these yield a smaller deposit on boiling in the flasks. It is also suitable for many bacteria and moulds, but for certain bacteria (*e.g.*, lactic acid bacteria) the sweet wort, without hops, is used, and this is also adapted for use with moulds. Amongst the artificial nutritive fluids for yeast, Pasteur's (1860) has an historical interest. The renowned scientist used this to upset Liebig's theory with regard to the indispensability of albuminoids for fermentation. It consists of 100 c.c. of water, 0.075 grammes of the ash of yeast, 10 grammes of sugar, and 1 grammme of ammonium tartrate. A good nutritive fluid can also be prepared from yeast decoction with

5 to 10 per cent. of sugar. Yeast decoction is an aqueous extract of yeast (about 1 litre of yeast to 2 litres of water, 'boiled under pressure), filtered, and either neutralised or rendered slightly alkaline with sodium carbonate or lime. For special research, compound liquids may be used containing sugar and the salts necessary for nutriment and normal growth of yeast, including potassium, magnesium, and calcium, phosphoric and sulphuric acids. An admirable means for preserving pure cultures of yeasts is the solution, first used by Pasteur, consisting of a 10 per cent. cane sugar solution.

Special nutritive liquids are also used for bacteriological investigation. Cohn's solution has historical interest, and its composition is as follows :—100 c.c. of water, 0.05 grammes mono-potassium phosphate, 0.05 grammes tri-potassium phosphate, 0.5 grammes crystallised magnesium sulphate, and 1 grammes ammonium tartrate. To-day a nutritive broth is chiefly used, prepared by steeping finely chopped beef for a few hours in water, and then boiling and filtering the liquor. The liquid is generally neutralised with soda, or rendered slightly alkaline, and after adding 1 per cent. of peptone and 0.5 per cent. of sodium chloride, it is again boiled, filtered hot, and finally sterilised in flasks or test tubes. Such an extract must obviously vary in composition, and in special cases resort may be had to an artificial nutritive liquid, free from albumen. We may quote that prepared by Voges and Proskauer, consisting of 1 litre of water, 5 grammes sodium chloride, 2 grammes disodium phosphate, 6 grammes ammonium lactate, and 4 grammes of asparagin.

A. Fischer's base consists of 0.1 per cent. of di-potassium phosphate, 0.02 per cent. magnesium sulphate, and 0.01 per cent. calcium chloride, dissolved in tap water. The solution is then added to peptone, or peptone and sugar, etc., according to the requirements of the particular species of bacteria. For the development of lactic acid bacteria (from milk) O. Jensen uses peptonised milk prepared by treating 1 litre of milk with 10 c.c. of hydrochloric acid and 2 grammes of pepsin. By keeping it in the thermostat and frequently shaking, the precipitated casein is redissolved ; the liquid is then neutralised, cleared with albumen, and sterilised at about 120° C.

For moulds, in addition to beer-wort, fruit decoctions and sugar solutions containing tartaric acid and tartrates are used, or, again, the complicated Raulin's liquid, which is also applicable to bacteria, and consists of—water 1,500 c.c., sugar 70 grammes, tartaric acid 4 grammes, ammonium nitrate 4 grammes, ammonium phosphate 0.6 gramme, potassium carbonate 0.6 gramme, magnesium carbonate 0.4 gramme, ammonium sulphate 0.25 gramme, zinc sulphate 0.07 gramme, ferrous sulphate 0.07 gramme, potassium silicate 0.07 gramme.

If solid nutrients are required, 5 to 10 per cent. of gelatine is added, or, in the case of cultures which are to be developed at or above 30° C., about 1½ per cent. of agar-agar, a jelly derived from salt water algae. For the cultivation of thermophilous bacteria at 60° to 70° C., Miquel uses carragheen moss instead of agar, in the proportion of 2 to 3½ per cent. Slices of potato sterilised in an autoclave are often used as a solid nutrient. Black bread makes an excellent solid substratum for moulds. For the cultivation of the nitrifying bacteria Winogradsky and Omelianski used gelatinous silicic acid.

For plate cultures of acid-forming bacteria (lactic acid and acetic acid bacteria) some litmus or, preferably, according to Beijerinck, carbonate of lime (finely precipitated chalk) is added. The gelatine thus acquires a motley appearance, but the colonies of acid bacteria are surrounded by a clear zone, because the acid dissolves the chalk. By the use of zinc carbonate in plate-cultures, the acetic acid bacteria form colonies and display clear zones, whereas the lactic acid bacteria are relatively sensitive to this salt, and their growth is inhibited.

Pasteur used liquids exclusively for his work on the ferments. Later, solid media became of great importance, and Koch laid the foundation for their application.

*Plate-cultures* are prepared by introducing the growth into the liquefied gelatine, and then pouring the mixture into a Petri dish. When the gelatine solidifies the individuals are separated throughout the mass, and, on development, they appear as colonies, visible to the naked eye. *Streak-cultures* are those in which a minute portion of the growth is intro-

duced on to the surface or into the upper layer of the solidified gelatine on a platinum spatula. *Stab-cultures* are those in which a fraction of the growth is introduced by an inoculating needle into a thick layer of solidified gelatine. *Giant colonies* are formed by pouring a drop of the inoculated liquid on to a stab in the solid gelatine.

#### 7. Preparation of the Pure Culture.

To prepare an absolutely pure culture, it is necessary to make sure by direct observation that the development begins with a single cell, and that this is so completely isolated that during the development no other cell can creep in and render the culture impure. If such a pure culture is required for experiments on a large scale or for actual fermentations, special rules must be observed in order that the absolutely pure growth at first developed shall be protected from every infection during its further growth in a succession of larger flasks. Care must, of course, be taken that the species is developed under the most favourable conditions to secure a vigorous and normal culture. The process in its later stages is described in another section. We are here concerned with the problem of securing the first absolutely pure culture as the point of departure for the mass culture.

The desideratum of direct observation presents difficulties in the case of the smallest micro-organisms—bacteria. Whilst it has long proved possible to directly observe single cells of yeasts and moulds on account of the size of their cells, this has not been the case with the great majority of bacteria. In such cases we must be content with methods which give a certain probability for preparing a pure culture. It is only quite recently that the technique has been sufficiently developed to allow of an approximately accurate solution of this problem.

Long before there was any attempt to work experimentally with absolutely pure mass cultures, experiments in the cultivation of micro-organisms had been undertaken with a purely botanical object, to discover what different forms a species may assume, and with this object the development of single cells was followed under the microscope.

As early as 1821, Ehrenberg observed the germination of the spores of certain fungi by careful observations of this kind. The propagation of yeast cells was observed by Mitscherlich (1843), Kützing (1851), and F. Schulze (1860), in the same way. A small quantity of top yeast was diluted with beer-wort until it contained only one or two yeast cells ; from a drop of this an ordinary preparation was made, the cover-glass was cemented on to the glass slide, to prevent the evaporation of the drop, and the development of the cell was watched under the microscope. Similar cultures were employed by Tulasne (1861) and de Bary (1866), in their famous researches on the germination of spores. A considerable improvement in the method was made by Brefeld during his detailed researches on mould, blight and mildew fungi, in which he followed the development of the mycelium until it, in its turn, again formed spores. The infection on the object glass was protected by means of a small shield of paper fastened on to the tube of the microscope, and this was afterwards converted into a moist chamber (1881), after Brefeld had recognised the danger of foreign germs penetrating into the cultures. He diluted the material with water, brought a drop containing a single germ on to the cover-glass, added some nutritive liquid containing gelatine, and placed the cover-glass, with the drop underneath, on to a glass ring (Böttcher's chamber), which was fastened to the object glass. As the apparatus and the nutritive liquid were sterilised, all the necessary conditions were fulfilled for carrying out a culture experiment without contamination. We may here see how improved methods of cultivation have led to the preparation of an absolutely pure culture. By the help of his cultures Brefeld made the interesting observation (1883) in quite a number of fungi—*e.g.*, the smut of wheat, the boil-blight of maize, etc.—that the conidia are able to propagate by direct budding, like yeast, without throwing out new seed-carriers.

A short survey follows of the different methods which have been applied for preparing pure cultures on the large scale.

(a) **Physiological Methods.**—At the earliest stage, attempts were made to reach the goal by calculating the probabilities,

and treating the whole growth, without condescending to isolate single cells.

The physiological methods—"the enriching process"—employed by Pasteur, Cohn, and others start with the fundamental idea that the various species occurring in a mixture will multiply unequally according to their different natures, when they are cultivated in one and the same nutritive liquid and at the same temperature, so that those species for which the conditions are unfavourable will be gradually suppressed by the one or more species for which the conditions are favourable. When the growth has developed under the selected conditions for quite a short time, a minute fraction is inoculated into the same nutritive liquid in a fresh vessel at the same temperature, and this process is repeated many times. Different liquids have been employed for such cultures from time to time; for instance, alkaline liquids for bacterial growths, acid liquids to free yeast growths from bacteria (lactic, tartaric, hydrofluoric acids, etc.). The weak point of all such methods is, that they *start from an unknown material*—namely, *the impure mixture*. It, is, therefore, impossible to know what results such a treatment will lead to, for we are not dealing with any true method, as contamination may take place at random. In fact, there is always the possibility that the weaker species are not destroyed, but merely checked and retarded, so that when the stronger species, after reaching their maximum development, become weaker, other species will have a chance of multiplying. This possibility also occurs when the growth is transferred to another substratum. Likewise, there is always the possibility that not one but two or more species thrive equally well in the liquid, and, consequently, develop to the same extent. Such, for instance, was the case with brewers' yeast before pure cultures were employed. This yeast often yielded several typically different species of "culture yeast," as they are termed, when examined by Hansen's method. The method given by Pasteur for the purification of brewers' yeast may be mentioned as a marked illustration of the dangers connected with the physiological method of treatment. The impure yeast-mass is introduced into a cane-sugar solution,

to which a small amount of tartaric acid has been added. The object of the method is to free the yeast from any disease germs with which it may be infected. Hansen's investigations have, however, proved that, even if the bacteria are suppressed or checked by this treatment, simultaneously the wild yeast, and among them those productive of diseases in beer, will develop abundantly, and in many cases the culture yeast, which it was intended to purify, is entirely suppressed. Even if there is primarily only a trace of the wild yeasts or "disease" yeasts, these are apt to develop to such an extent by Pasteur's treatment that they may eventually form the predominant part of the yeast-mass.

The use of hydrofluoric acid or its compounds, such as ammonium fluoride, for the purpose of purifying an impure yeast—brewers' or distillers' yeast—as proposed by Effront, is liable to lead to the same dangers as the use of tartaric acid. Methodical experiments made by Holm and the author have shown that by treating impure yeast according to Effront's process, the growth of wild yeast and *Mycoderma* species is forced more than that of the culture yeast; they have also shown that such a dangerous species as *Bacterium aceti* is in many cases not suppressed at all by the treatment in question, but, on the contrary, multiplies more rapidly in presence of hydrofluoric acid or fluorides.

If, now, we ask, whether it is advisable to employ any of the various methods mentioned above for the purification of an unknown and impure yeast-mass, the answer must be in the negative; and this will be the case whether the culture is intended for purely scientific or for industrial purposes, for the danger will never be excluded that in prolonged cultivation other species than the one desired will gain the supremacy. The starting point being uncertain, it necessarily follows that the result must be so too. In fact, all such methods must now be regarded as antiquated, and as complete failures. Nevertheless, they may possibly be used in isolated cases before proceeding to the preparation of a pure culture. In this way it is possible by suitable treatment of the impure material to secure a preponderance of the group of the desired species in the mixture, so that a pure cultivation

is facilitated. Thus the treatment described with tartaric acid or hydrofluoric acid gradually converts the mixture into a growth of wild yeasts. If a mass of yeast is strongly contaminated with bacteria, a cultivation at very low temperatures may possibly suppress the bacteria if the yeast are able to develop under these conditions. If it is desired to obtain a pure culture of the lactic acid bacteria from a mash, the material may be prepared by keeping it a short time at 50° to 55° C. At this high temperature many bacteria cannot thrive, whilst certain species of lactic acid bacteria can stand a high degree of heat, and thus spread throughout the material. In the same way in the cultivation of film-forming bacteria, such as acetic acid bacteria, the growth may undergo a preliminary purification by repeated inoculation of the film in fresh liquids. This process was used by Pasteur in his researches on acetic acid bacteria. To make an approximate separation of a large and small species of yeast in a mixture we may resort to decantation or filtration through a medium which will allow the small cells to pass.

It is common to all these methods that with more or less luck it is possible to bring about the preponderance of one or more groups of micro-organisms in a mixture, but it is obviously impossible to obtain in this way the exclusive presence of one particular species.

(b) **Dilution Methods.**—The second group of methods employed for physiological purposes embraces the dilution methods, or "fractional cultivation," the principle of which is to dilute the material to such a degree that it is ultimately possible to isolate a single cell. Brefeld used the dilution process for his botanical investigations of moulds, where he was able, owing to the size of the cells, to insure that only a single cell was present in a small drop of water in the moist chamber. He added sterile nutritive fluid, and observed the growth of the cell. Pasteur utilised air (*Études sur la bière*, 1876) as a diluting medium for preparing pure cultures. He started from the fact that if nutritive liquids are exposed to the action of air, fermentation takes place, excited by the germs which fall on to the surface. To isolate single germs from the yeast mass, he proceeded as follows:—A small

quantity of yeast was dried and ground with powdered gypsum. The fine dust was thrown into the air at as great a height as possible, and whilst the particles were floating down, a series of vacuum flasks were opened. Thus some of the yeast cells which were finely distributed throughout the dust cloud might penetrate singly into some of the flasks.

The first application of the dilution method to bacteria was made by Lister (1878). To prepare pure cultures of lactic acid bacteria he first determined microscopically the number of bacteria in a minute drop of sour milk, counting them in several fields of the preparation, and thus calculating their whole number. He then estimated the amount of sterilised water it was necessary to add so that after dilution there would be on an average less than one bacterium in each drop. With five such drops he inoculated in one case five glasses containing boiled milk. The result was that the milk in one of these coagulated, showing that it contained *Bacterium lactis*, whilst the four other glasses remained unaltered, and did not show the presence of bacteria. The same method was subsequently employed by Nägeli and Fitz (1882).

In comparison with the physiological methods the dilution method now described is a distinct advance; indeed we have thus approached much nearer to the goal. On the other hand, it is clear that, even if the dilution is carried as far as in the case mentioned, in which only one of several flasks shows development, it is not yet proved that this one flask has received only one germ. Thus, there is still great uncertainty, even in cases where the individuals with which we are working can be counted. Moreover, it is extremely difficult to count individual bacteria, and often, indeed, quite impossible. In all cases the accuracy of such calculations is very questionable. Thus, the problem remains to be solved: How are we to distinguish the flasks which have only received one cell from those which, notwithstanding calculation, have been infected with several cells? For the bacteria, no means have as yet been found of solving this difficulty.

In the case of yeast the process was further developed by Hansen (1881). He employed dilution with water, in the following manner:—The yeast developed in the flask is diluted

to a given proportion with sterilised water, and after vigorous shaking, the number of cells in a small drop of the liquid is determined. The counting, in this case, is easily carried out by transferring a drop to a cover-glass, on the centre of which some small squares are engraved, which form a starting point for the eye, and this is then attached to a moist chamber (Fig. 4); the drop must not be allowed to extend beyond the limits of the squares. The cells present in the drop are then counted. Suppose, for instance, that 10 cells are found; a drop of similar size is transferred from the liquid, which must first be shaken vigorously, to a flask containing a known volume—*e.g.*, 20 c.c. of sterilised water. This flask, then, will in all likelihood contain about 10 cells. If it is now vigorously shaken for some time until the cells are equally distributed in the water, and 1 c.c. of the liquid introduced into each of 20 flasks containing nutritive liquid, then by calculation half of these 20 flasks should receive one cell each. If the infected flask is strongly shaken and then allowed to stand, the single cells sink and remain on the bottom. It is evident that if a flask contains three cells, they will, in the great majority of cases, be separated by the vigorous shaking, and be deposited in three distinct places on the bottom. After some days, if the flask is raised carefully, it will be observed that one or more white specks have formed on the bottom of the flask. If only one such speck is found, then in all probability the flask has only received a single cell.

It was by this method that Hansen prepared all his earliest pure cultures, with which he carried out his fundamental researches on alcoholic ferment.

**Solid** nutrient media have also been employed for the preparation of pure cultures by the dilution method. The foundation of such methods was laid by Schroeter (1872), who, in his researches on pigment-bacteria, employed slices of potato as a nutrient. He had observed that when such slices had been exposed for some time to the air, specks or drops of different form and colour made their appearance. Each of these specks usually contained one species of micro-organism.

Koch considerably developed and improved this method.

He at first prepared his pure cultures by means of streak infections in nutrient gelatine. He afterwards devised a far better method, the plate-culture method (1883). The process is as follows:—A trace of the crude culture is transferred to a large proportion of sterilised water. From this a small quantity is transferred to a test-tube containing, for instance, a mixture of meat-broth and gelatine warmed to 30° C. The tube is shaken in order to distribute the germs, and the contents poured on to a large glass plate, which is then covered with a bell-jar. The gelatine quickly sets and the germs are enclosed in the solid mass. In a few days they develop to colonies—dots or specks which are visible to the naked eye. The purity of the bacterial growths in the gelatine is ascertained, according to Koch, partly by their appearance.

An improvement in the method consists in the use of glass dishes with lids instead of glass plates, the Petri dishes (introduced by Salomonsen), into which the liquefied gelatine is poured: or the “roll-tubes” of Esmarch may be used, prepared by continuously rotating a test-tube round its longer axis until the inoculated gelatine has set in the tube, so that the whole of the inner surface is covered.

When species are being developed which require a high temperature (at which gelatine would be liquefied), plates are made of agar, or of agar and gelatine. The growth can be mixed with the liquefied material, or else spread over the surface of the solid, either by strokes of a platinum pencil, or by stabs with an inoculated needle. After selecting colonies, which appear to be pure, from a plate prepared in any one of these ways, a new plate-culture may be prepared from one colony. If all the colonies that develop on this plate are pure, it is probable that we are dealing with a pure culture.\*

When regarded more closely it will be seen, however, that there is no essential difference between the distribution of

\* Great importance is ascribed to the appearance of colonies of bacteria on gelatine, to their colour, shape, the nature of the edge, etc., and whether they liquefy gelatine or not. Other characters of the gelatine cultures are taken from the streak- and stab-cultures. According to Hansen, colonies of many species of yeast on gelatine plates exhibit characters of great value. The giant cultures of Lindner are also used.

the germs in liquefied gelatine, and Lister's method of dilution by means of liquids. The same uncertainty is always present ; neither the macroscopical observation of the appearance of the colony nor the microscopical examination of its contents gives any surety of its only containing one species.

The only possibility of securing a really pure culture in the gelatine consists in the direct observation of one individual germ and its development.

Hansen did this for yeast by using Böttcher's moist chamber. The lower side of the cover-glass is covered with a layer of wort-gelatine, in which the yeast cells are distributed. On account of the size of the latter, it is possible to see whether a single cell lies so wide apart from other cells that the colony developed from it will form a pure culture.

The chamber is then either allowed to remain under the

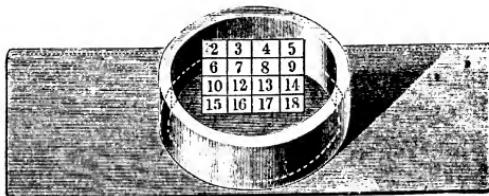


Fig. 12.—Jörgensen's moist chamber with etched squares and numbers.

microscope, in order that the propagation of the germs may be directly followed, or the positions of well isolated germs are marked, either by dividing the glass-cover into small squares, or by means of the object marker, and the apparatus is placed in the incubator until the colonies are fully developed. The cover-glass is then lifted off and placed under a bell-jar, so that the gelatine layer is turned upwards, and the colonies are transferred into flasks. In the author's laboratory moist chambers like that represented in Fig. 12 are used, the cover-glass being etched with 16 squares and numbers. The situation of the cells is then marked on a sketch plan, which shows all the etched numbers and squares. The author has altered the process by cementing the cover-glass on to the glass ring, and fastening the latter to the object glass with vaseline. To remove the chamber, the ring is lifted off, and this is a

more convenient and more certain process than lifting the cover-glass, for it is possible to transfer the colonies without inverting it. On one cover-glass there may be 50 to 60 well isolated germs. When the colonies are conveyed to the flasks by means of a small piece of platinum or copper wire, which has been previously ignited and cooled, the culture is momentarily in the air, and is then exposed to contamination. But the danger of contamination at this, the single weak point, is reduced to an insignificant minimum, and disappears if the operation is performed in a small enclosed sterile space: for instance, in a small cupboard with glass sides sufficiently large to admit the apparatus and the operator's hands. In this way the transference of the colonies is effected with all possible security. From the first flask the culture can be transferred without contamination to a continually increasing number of larger flasks.

For the pure cultivation of brewery, distillery, and wine yeasts, vigorous cells must be conveyed to the gelatine in the moist chamber. According to J. C. Holm, on an average only about 4 per cent. of the inoculated cells do not develop, whilst from a growth of yeast that is taken at the end of the fermentation, in which the cells are weakened, about 25 per cent. do not develop. It is usually preferable to convey a small average sample of the yeast into wort or must, and then to use the very young growth, which is developed when the first trace of fermentation is observed. To decide whether any of the selected yeasts are of value for industrial purposes, a large number of cells must be isolated, as indicated by the author as early as 1885. After years of experience, it has proved impossible to speak of a preponderant species or race from which any individual can be chosen. The single type or species contains within itself so many varieties which have come to development under the conditions existing in practice, that a careful choice must be made from these. A thorough study of a type by means of comparative experiments will show which of the cultures is of the greatest value in practice.

As early as 1883, Koch's method of plate-culture was tested by Hansen. He prepared a mixture of two species of

yeast which can be distinguished from each other microscopically—viz., *Saccharomyces apiculatus*, and a species of the group *S. cerevisiae*. This mixture was introduced into wort-gelatine, and after shaking was poured on to a glass plate. Of the specks formed, about one-half contained one species exclusively, the other half the other species, and in one of the specks both species were found.

A similar control was carried out for bacteria by Miquel (1888), who introduced 100 colonies from a plate-culture obtained in an air analysis into 100 flasks containing meat-broth with peptone. The examination of the growths developed in the flasks showed that they contained 134 different species of micro-organisms. This evidently depends upon the fact that it is very difficult, and often quite impossible, to separate all germs of bacteria and other organisms from each other by shaking the gelatine mixture.

Holm has subjected the method to a thorough analysis (1891), in the case of a large number of yeast species, absolutely pure cultures of which were prepared by the Hansen method. The result of 23 series of experiments with different mixtures was that only in a single case were 100 colonies developed from 100 cells. In all the other series the method proved faulty. In the most unfavourable case 100 colonies were formed from 135 cells, and the average number obtained was 100 colonies from 108 cells. This proves the plate method to be defective also in the case of yeast.

A modification of Brefeld's culture of a single cell in a hanging drop is that known as the drop culture, introduced by P. Lindner in 1893. It consists in conveying to a cover-glass a number of small drops of a diluted culture in a nutritive liquid by means of a mapping pen. The cover-glass is fastened by a ring of vaseline on to a hollow-ground object glass, and those drops are noted that contain only one cell. Care must, therefore, be taken that the drops do not flow together before the pure culture is conveyed to a flask.

Burri attempted to solve the problem of preparing pure cultures of bacteria under direct observation of single cells by the help of his Indian ink point culture. He dilutes ordinary liquid Indian ink with water in the proportion of 1 to 10, and

after sterilisation infects with the bacteria, and then dilutes to such an extent that small drops of 0.1 to 0.2 mm. diameter contain on an average a single germ. Such drops are placed at suitable distances with a mapping pen on the surface of a layer of nutrient gelatine, where they immediately evaporate, and are then protected by flamed cover-glasses. Much smaller drops can be deposited on gelatine than on a cover-glass. The contents of these specks can then be controlled under a high power, and those noted which contain a single germ, a process that is rendered easier by the fact that the bacteria appear clear on a greyish-brown ground. If they can grow in gelatine the development is allowed to continue, but if they require high temperatures they may be conveyed to an agar plate by cautiously raising the cover-glass, and as the speck of ink is more firmly fastened to the glass than to the gelatine, the germ is carried with the glass, so that it can be conveyed along with this to the agar plate. After removing the germ from the gelatine plate a drop of nutritive liquid can be placed on the ink fleck of the cover-glass, and so the germ may be developed in a liquid. The process can also be used for the cultivation of anaërobic bacteria.

Anaërobic bacteria demand special methods of cultivation, in which the atmospheric oxygen must be removed both from the substratum and from the space in which the bacteria are growing. Pure cultivations may be carried out in nutrient gelatine or agar, in tubes filled almost to the top, the bacteria growing in the bottom layers.

A still better process consists in removing the air from the test tubes by means of an air pump, whilst the glass is immersed in water at 30° to 35° C., after which it is hermetically sealed. Another method is to remove the air with a current of hydrogen. This is conveniently carried out in the following way (*Fränkel*) :—A wide test-tube is fitted with a stopper with two holes carrying two glass tubes, one of which reaches to the bottom of the test-tube ; the other terminates just below the stopper. When the vessel has been covered with nutrient gelatine or the like, and sterilised, it is inoculated with the growth, and a stream of hydrogen is passed through the long tube. The tube is sealed up as soon as the air is completely

driven out. The stopper is sealed with paraffin wax, and if gelatine or agar is being used the test-tube is rotated round its longer axis until the material has solidified. The bacteria develop slowly on the inner surface of the glass.

The cultivation can also be carried out by utilising a substance that will absorb oxygen—*e.g.*, pyrogallic acid (1 gramme of the dry reagent in 10 c.c. of a one-tenth normal potash solution). To carry this out the open test-tube or plate-culture is placed in a larger air-tight test tube or vessel containing the reagent. The absorption of oxygen goes on slowly, and requires 24 hours or more. The culture may also be covered with paraffin, vaseline, oil, plates of mica, etc.

Reducing substances like grape sugar, especially in an alkaline solution, or minute quantities of formic acid or sodium indigo sulphonate may be added to the nutritive substance, in order to favour the growth of anaërobies.

If it is wished to ascertain with certainty, in using one of these processes, when all oxygen has disappeared, a concentrated alcoholic solution of methylene blue may be used as an indicator. A few drops are added to the nutrient, and as soon as the last trace of oxygen is absorbed or removed the indicator will be entirely decolourised. The same applies to the addition of indigo carmine (neutral sodium indigo sulphonate).

#### 8. Counting the Yeast Cells.

The multiplying capacity of the yeast cells can be estimated by directly counting the cells that are present in a given volume of the liquid at different stages of the fermentation. Experiments on these lines have been undertaken especially by Delbrück, Durst, Hansen, Hayduck, and Pedersen, whilst Fitz has applied the method of counting to bacteria.

The counting is performed by means of an apparatus constructed by Hayem and Nachet, and by C. Zeiss (Fig. 13), which was first employed for counting the corpuscles of blood (hence termed *hæmatimeter*). Panum was the first to employ this apparatus for counting micro-organisms, in order to

determine their multiplying capacity. The haemmatimeter consists, as shown in the diagram, of an object glass on which a cover-glass of known thickness (0.2 mm.) is cemented, and from the centre of which a disc has been cut out. A small drop of the liquid containing the cells is brought into the cavity thus formed, a second cover-glass is placed over the opening, and thus rests on the cemented and perforated cover-glass. The drop of liquid must not be so large that the pressure of the cover-glass causes it to flow out from the enclosed space, yet it must be high enough to be in contact with the cover-glass. The thickness of the layer of liquid is then known. In order to determine the other two dimensions, and thus be able to work with a given volume of liquid, one of the well-known forms of micrometer is introduced into the eye-piece of the microscope. It may consist of a thin piece

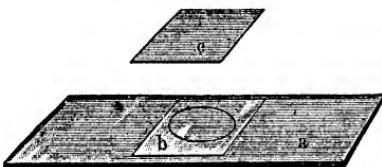


Fig. 13.—Haemmatimeter—*a*, object-glass; *b*, cemented cover-glass with circular opening; *c*, cover-glass.

of glass on which 16 small squares are engraved. The actual value of each of these squares is known when a given system of lenses is employed, and thus, when the square is projected on the object, a small prism of known volume is defined. In certain cases it may be more expedient to make use of an appliance constructed by Zeiss, of Jena, from the instructions of Thoma, which consists of a fine system of squares of known size, engraved on the object-glass itself at the bottom of the cavity. This also improves the microscopical definition of the cells which are on the bottom of the chamber.

When it is merely desired to determine the rapidity with which the cells multiply, or to make repeated observations of the number of cells in the same volume, it is quite superfluous to determine its size; it is simply necessary to work always with the same volume.

The sample taken should always be a fair average. In most cases it must be diluted and thoroughly agitated for a long time, in order to obtain an equal distribution of the cells; the specific gravity of the liquid must also be such that it will allow the cells to remain suspended in it for a short time. A small drop is then withdrawn in a capillary tube, transferred to the counting apparatus, and covered with the cover-glass. The apparatus is allowed to remain at rest for some time, in order that the cells may settle to the bottom of the enclosed space, and on this account the specific gravity of the liquid must not be greater than will allow this to take place in a convenient time. Both these requirements are generally satisfied by the wort employed in breweries.

If it is found that the determinate volume contains too many cells to be counted with certainty, the liquid must be diluted. This may be advisable for other reasons, partly to prevent the formation of froth, which may otherwise form abundantly from the violent agitation, and partly to isolate the single cells which frequently cluster as colonies in the wort, and are not always separated by shaking. Finally, it is necessary, whilst the counting is going on, to arrest the development of the yeast cells in the sample.

Hansen found that dilute sulphuric acid (1 to 10) on the whole answers these requirements; hydrochloric acid, ammonia and caustic soda may also be used, but they are not so good. If very great dilution is required, distilled water may be added, after the addition of one to two volumes of dilute sulphuric acid.

When the different volumes of liquid are measured with accuracy, and particular care taken that the cells are thoroughly distributed by vigorous and prolonged shaking, the determination can be made with great accuracy. Two similar dilutions must always be made, and samples taken from each for counting. As a matter of course, experiments must also be made to determine the number of the small squares, the cell contents of which must be counted to arrive at a true average. Such counting and determination of the average numbers is continued until the number finally obtained is found to have no

further influence on the average value. The number of countings necessary, and the accuracy generally, depends on the experience and care of the observer. Hansen found that, as a general rule, it was sufficient to count the cells in 48 to 64 small squares.

## CHAPTER II.

**BIOLOGICAL EXAMINATION OF AIR AND WATER.**

THE investigations into spontaneous generation already referred to naturally led to the study of the organisms in air, and after Pasteur, in particular, had demonstrated that air contained, not bacteria only, but also fungi giving rise to alcoholic fermentation, air analyses acquired an interest for the zymophysiologist, and for the fermentation industry. Such comprehensive researches are now available that it has been possible to arrive at an idea of the biological composition of the air on a large scale, and to form a judgment of these conditions in relation to the brewing industry. At first, when it became known that crowds of living germs, capable of development, could occur even in very small volumes of air, there was a natural inclination to exaggerate their effect in practice, and to attribute any excessive growth of disease germs in a fermentation to the direct influence of the air.

An exhaustive study of the conditions occurring in practice, carried out in recent years under systematic biological control, has shown that this influence had been exaggerated, and that it is possible, even where an air analysis has shown the presence of numerous germs, capable of producing disease in a fermenting liquid, to suppress the partly dried and weakened germs falling into the liquor by the addition of the excessive number of yeast cells contained in the pitching yeast.

Large growths of disease-producing organisms were only found in practice if they had been allowed to develop on certain infected areas. The germs in the air are thus only indirectly the cause of disturbances in practice, and under normal conditions can seldom be of importance.

The majority of air analyses have been undertaken with a view of throwing light on the obscurity which surrounds most contagious diseases, nearly all of which are, as is well known, attributable to the agency of micro-organisms. With regard to the organisms of fermentation, these have been investigated by Pasteur, and, later, especially by Hansen. The French *savant* stated that, whilst these germs are always floating about in the air, *they are present in much larger quantities in the dust which settles on the vessels and apparatus employed*. The actual fungi giving rise to alcoholic fermentation are present in comparatively small numbers in the air, whilst the germs of moulds are more frequent; he further showed, as was subsequently done by Tyndall, that the germ-contents of the air vary both with regard to quantity and species. These results were obtained by exposing beer-wort, wine-must, or yeast-water containing sugar, in open, shallow dishes, at different places, and examining their contents after some time for microscopical germs. Pasteur also employed for this purpose the so-called vacuum flasks, containing nutritive liquids and rarefied air. On opening the flask a sample of germ-laden air could be drawn in.

The most important air analyses undertaken in recent years are, without doubt, those carried out by Miquel, the director of the laboratory specially arranged for this purpose at Montsouris, near Paris. His fellow-worker, Freudenreich, has also made valuable contributions to our knowledge of this subject.

Miquel performed his first experiments with a so-called Aëroscope (Fig. 14), which is constructed in the following manner:—A bell-shaped vessel, A, is provided with a tube, C, through which air can be aspirated. A hollow cone, shown in the left-hand figure, is screwed into the bottom of A; the mouth of the cone, B, points downwards; in the apex, D, of this cone there is a very fine opening through which the air is aspirated, and immediately over this opening is fixed a thin glass plate covered with a mixture of glycerine and glucose. The particles carried in by the air settle to some extent on the viscous mixture. The intercepted micro-organisms are distributed as equally as possible on the glass plate, and counted under the

microscope. This method is defective in so far as it gives no information on the most important point—namely, which and how many of the intercepted germs are actually capable of development.

In order to determine the number of germs capable of development, and also their nature, Miquel employs the following apparatus (Fig. 15):—The flask *A* has fused into it a tube, *R*, tapering below and nearly reaching to the bottom; the upper end of this is fitted with a ground cap, *H*, provided with a narrow filter-tube containing sterilised cotton-wool, asbestos, or glass-wool, *as*. On one side of the flask is a tube, *Asp*, which is constricted in the middle, and is provided with two cotton-wool plugs, *w'* and *w*. On the other side is another



Fig. 14.—Aëroscope.

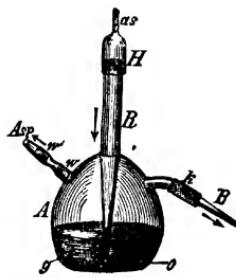
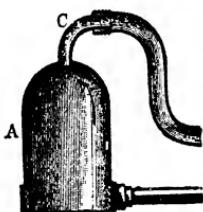


Fig. 15.—Miquel's apparatus for air analysis.

glass tube connected by rubber, *k*, with the tube *B*, which is drawn out to a point, and closed by fusing the end. The flask is partly filled with distilled water, and the whole apparatus sterilised. When the apparatus is to be used, the tube *Asp* is connected with an aspirator (e.g., a bottle filled with water and provided with an outlet cock); the cap *H* is taken off, and the air then passes, bubble by bubble, through the opening *o*, through the water *g*, and out through the cotton-wool plugs of the tube *Asp*. Since all the germs in the air are not retained by the water when the air-bubbles ascend through the latter, the cotton-wool plug *w* is intended to catch those which get past the water. When the experiment is finished, the cap *H* is replaced over the tube *R*. By blowing through *Asp*, the liquid is made to ascend in *R*, in order that

any germs which may have settled on the walls of the tube may be washed down into the liquid. Then, by blowing with greater force, the inner cotton-wool plug *w* is driven down into the liquid, and its germs shaken off into the latter. After sterilising the thin tube *B* in a flame, the point is nipped off, and the liquid is now—by blowing through *Asp*—transferred, drop by drop, into a large number of flasks containing sterilised broth.

The main object then is, by means of preparatory experiments, to obtain such a dilution of the air-infected water that a considerable proportion of the small flasks (one-half for example) remain sterile after inoculation ; or reveal samples of the water may be diluted to different degrees, and a series of flasks inoculated from each dilution ("fractional cultivation"). If a large number of the flasks show no development of organisms, there is a certain probability that in each of the remaining flasks in which growths have developed, only one germ has been sown. A simple calculation will then show how many germs capable of development in the medium employed were present in the volume of air aspirated through the original flask.

By these methods of investigation Miquel found that similar volumes of air in the same locality contained at different times a varying number of bacteria. Continued rain purifies the air from bacteria to a marked extent, and their number continually diminishes as long as the earth is moist ; but when the ground dries, it gradually increases again. Thus in the dry seasons of the year the number of bacteria is usually the greatest, whilst the moulds, which thrive best in moisture, and carry spore-bearing hyphae, which project upwards, are most abundant during the wet seasons. The purest air is found in the winter time ; the air of towns is less pure than that of the country ; germ-free, or nearly germ-free air is found at sea and on high mountains. In certain places—hospitals, for instance—the air has been found to be very rich in bacteria ; in one case even fifty times richer than the air in the garden at Montsouris.

An entirely different method for determining the organisms contained in air is that employed in Koch's laboratory, and

more completely developed by Hesse. A glass tube, about 1 meter long and 4 to 5 cm. wide, is closed at one end with a perforated india-rubber membrane, over which another non-perforated cap is bound. A little liquefied nutrient gelatine is then poured into the tube, after which the other end is closed with an india-rubber stopper, through which passes a glass tube plugged with cotton-wool. The whole apparatus is then heated sufficiently to render it sterile, after which the tube is placed in a horizontal position, so that the gelatine sets in a layer in its lower part. When the air is to be examined, the outer india-rubber cap is removed, and air slowly drawn through the tube. The germs contained in the air settle down on the gelatine, and after the aspiration is concluded the tube is again closed and placed in the incubator, where some of the germs produce visible colonies, which are easily counted. The results show that with a sufficiently slow current of air, the bacteria, which are often floating about in the air in larger or smaller aggregations, frequently clinging to dust-particles, small fibres, or splinters, settle sooner than the mould-spores; so that the gelatine in the fore part of the tube generally showed a preponderance of the bacteria colonies, whilst the mould-spores developed further on.

Miquel's method is to allow the air to pass through a hollow cylinder of solidified gelatine, in which the germs are retained.

Hueppe, v. Schlen, and others use liquid gelatine for air analyses, the air being aspirated through the gelatine, after which the latter is poured on to glass plates.

Frankland, Miquel, Petri, and Ficker use porous solid substances for the filtration of air for analytical purposes; as, for example, powdered glass, glass-wool, sand, sugar, etc. The sand-filter employed by Petri is 3 cm. long and 1.8 cm. wide. It is packed with sand, previously ignited, the size of the grains being from 0.25 to 0.5 mm. Two such sand filters are placed one behind the other in a glass tube. In the first filter all the dust-particles containing germs should be retained, whilst the second filter serves as a control. The sand charged with germs is distributed in shallow glass dishes and covered with liquid gelatine. The germs accompanying the dust-particles will then form colonies in the gelatine.

When samples of the air contents are to be sent from one place to another, these air filters will answer the purpose. On receipt of a sample, the sand may be washed into gelatine or, preferably, into sterilised water. After vigorously agitating the water, it is added in drops to flasks containing nutritive liquid, or it may be used in plate-cultures.

When samples of air are to be sent to the author's laboratory short cylindrical glasses are used, having india-rubber stoppers, which project well beyond the mouth of the glass. The latter are half-filled with sterilised water or with the nutritive liquid in actual use. When the glass is opened at its destination the stopper is placed by it with the wet end turned upwards, care being taken, of course, that this is not touched. A suitable time having elapsed, the stopper is replaced and tied down.

Miquel has raised an objection to the employment of gelatine plates for this purpose, based upon numerous experiments. He asserts that many bacteria, when exposed to a temperature of 20° to 22° C., require a fortnight's incubation before developing distinct colonies in gelatine; on the other hand, there are species which very soon liquefy the gelatine, thus rendering further observation impossible. The same is the case with the moulds, which often spread all over the plate in a few days. Thus, it becomes necessary to count the colonies at so early a stage that many of them are not yet visible. An additional drawback to the gelatine plates is, that the development cannot take place at a temperature higher than 23° to 24° C., otherwise the gelatine will liquefy, but many species of bacteria present a characteristic development only at considerably higher temperatures. Other species, moreover, do not develop in gelatine at all, but only in liquids. Finally, it is urged as a very material objection to the gelatine plates that many of the colonies consist of several species. Miquel proved this by introducing the colonies, one by one, into meat decoction with peptone, and then again preparing plates from these growths. This is in part due to the fact that the bacteria, as shown by Petri, often occur in aggregates in the air, and these will either fall directly on to the gelatine plate or become mixed in the liquid gelatine, where it would

be very difficult to separate the individuals from each other by agitation.

• E. C. Hansen's investigations of the air were made between 1878 and 1882. His main object was to throw light on questions affecting the fermentation industries. As is well known, his researches on *Saccharomyces apiculatus* (1880) were partly based on work of this nature. Since the question concerned the organisms which occur in brewing operations, the choice of a nutritive liquid was easily determined—namely, wort as ordinarily employed in breweries. The apparatus used consisted either of Erlenmeyer flasks closed with several layers of sterilised filter paper, the contents of which were boiled for a certain time, or of vessels similar to Pasteur's vacuum flasks, the necks of which were drawn out to a fine point, and closed with sealing-wax while the contents were boiling. A little below the point a scratch was made with a file, so that the point might be easily broken off when it was desired to admit air.

When these flasks had been filled with the air of the locality to be examined, they were again closed with sealing-wax and thoroughly shaken in order to mix the contents of the infiltrated air with the liquid. The flasks were then put aside for a longer or shorter time, lasting in some cases for six weeks, and their contents examined under the microscope.

In these investigations Hansen often found that the wort remained bright and apparently unchanged, even although a growth had taken place. Hence, the examination with the naked eye alone cannot be relied on. He names the following forms which, when present in a feeble state of growth, cannot be detected macroscopically :—*Aspergillus*, *Mucor*, *Penicillium*, *Cladosporium*, *Bacterium aceti* and *Pasteurianum*, and *Mycoderma cerevisiae*. Even when these micro-organisms have formed vigorous growths, the wort used has remained bright.

It was further shown that pure cultures may often be obtained by the use of these flasks, when only one species gained access to the flask along with the air. It very seldom happened that three or four species were found in the same vessel. This arises from the fact that only a very small volume of air enters each flask. The advantages of this are evident :—

A true knowledge of these germs can only be obtained when they have developed ; in cases where several germs penetrate into the same flask, the strongest germ would by its growth, in all probability, prevent the development of the others, so that these would not be detected in a subsequent examination. At the same time this method necessitates the opening of a large number of flasks, which makes the operation cumbersome and costly. As the flasks only show what was present in the air at the moment of opening, Erlenmeyer flasks were also used to give supplementary information, for which purpose they were allowed to remain in the same locality for a long time, in some cases as long as 48 hours.

After these preliminary remarks, we will give a brief summary of the results obtained by Hansen.

He confirmed the statement made by Pasteur and Miquel that the air at adjacent places, and at the same time, may contain different numbers and different species of organisms ; and he found that this holds good for adjacent parts of one garden. Hansen mentions, amongst other factors determining the distribution of micro-organisms, that those forms, for instance, which in the first half of July commonly occurred under the cherry trees in the garden, were in the latter half of the same month entirely absent from this locality ; further, that organisms which at one time could be found under the cherry trees, but not under the vines, were to be found later only under the latter. As a proof of the inequality of distribution of the organisms, he showed that flasks opened in the same place in the same series of experiments often had the most diverse contents.

The experiments with vacuum flasks have further taught us that the micro-organisms of the air often occur in groups or clouds, with intermediate spaces, which are either germ-free or only contain a few isolated germs. As the organisms are not generated in the air, but on the earth and on fruit, it follows that their presence in the air must be dependent on the condition of the surface of the ground and of the fruit, which again depends to some extent on the weather.

Hansen's numerous analyses have further proved that the *Saccharomyces* comparatively seldom occur in the dust of

the air.' Their number in the open air increases from June to August to such an extent that flasks at the end of August and the beginning of September are frequently infected with these organisms, after which a decrease takes place. The *Saccharomyces* which are found at other times of the year in the atmosphere may be regarded as unimportant in numbers and accidental in occurrence. As most species of the *Saccharomyces* have in all probability—like *Saccharomyces apiculatus*—their winter quarters in the earth, and their propagation areas on sweet succulent fruit, the latter must be considered as the most important source of contamination. During the same season bacteria are also found in the largest numbers. This constitutes a real danger in technical operations, since wort, when spread in a thin layer on the open coolers, is exposed to a source of contamination from the atmospheric germs.

Bacteria are found in the flasks in somewhat greater number than the *Saccharomyces*, whilst the moulds occur in still greater numbers. Amongst the latter *Cladosporium* and *Dematiuim* are especially prevalent in gardens, and after these *Penicillium*; whilst *Botrytis*, *Mucor*, and *Oidium* occur less frequently.

After Hansen had thus demonstrated which of the micro-organisms existing in the open air are capable of developing in flasks with sterilised wort, he proceeded to communicate the results of his examination of different parts of the brewery.

When grains (draff) are allowed to stand in the open air, they evolve, as is well known, acid vapours, and since they always harbour a rich growth of bacteria when they remain exposed for a short time, the question naturally presents itself, what is the condition of the air in the neighbourhood of heaps of grain? It was found that only 30 per cent. of the flasks opened in these areas became infected, and of these 3.6 per cent. with *Saccharomyces* and 2.4 per cent. with bacteria, whilst parallel experiments in the garden gave a contamination of about 44 per cent., of which 8.5 per cent. were bacteria. The air near the grains thus contained fewer bacteria than the air in the garden. The most abundant contamination was that of moulds, as in all other localities.

After a thorough examination Hansen came to the conclusion that, without doubt, scarcely a single organism which entered the flasks proceeded from the grains. The result tends to show that in this, as in other cases, air does not take up organisms from moist surfaces.

This, however, must not be misunderstood to mean that grains may be allowed to accumulate, without risk, and that after removal, the residue may be exposed to the weather. It is clear that this would constitute a great danger. When the remains become dry and are blown about in the air as dust, masses of bacterial germs will be carried up at the same time, and will, without doubt, constitute a source of frequent bacterial contamination. For this reason, places where grains have remained for any length of time must be washed with lime-water or, preferably, with chloride of lime.\*

In a corridor which led to a room where the barley was turned, the flasks always received a greater contamination than anywhere else; bacteria especially were found in them in great abundance.

On the malt floors the condition of the air was also characteristic; it always contained a very strong contingent of mould spores. In the case in question these consisted of *Eurotium aspergillus*, which was otherwise rare. On the malt itself, as usual, *Penicillium glaucum* occurred most frequently.

The greatest interest, however, attaches to the examination of the different fermenting-rooms, partly in the "Old Carlsberg" brewery and partly in the brewery "N." In the former the air contained fewer organisms than in any of the rooms examined during the whole research; in the fermenting-cellars of the brewery "N," on the contrary, a large number of flasks (55, 75 to 100 per cent.) were infected. The organisms which occurred in the air of these cellars were—*Saccharomyces cerevisiae*, *Mycoderma cerevisiae*, *S. Pastorianus*, *S. ellipsoideus*, *Torula*, and other yeast-like cells; further, *Penicillium*, *Dematiium*, *Cladosporium*, and rod bacteria.

\* The germs are not killed during the treatment of the grain in drying machines. Such forms of apparatus, therefore, constitute a very great danger in the brewery, since dust-clouds of bacteria may be transported from the dried grains to the open coolers or into the fermentation vessels.

Hansen was thus enabled, by a favourable chance, to contrast the state of the air in the most important part of these two breweries ; on the one hand an almost germ-free air, on the other hand an atmosphere teeming with germs. That the product of the latter place must have been affected by the atmospheric conditions then existing admits of no doubt, and this brings us face to face with one of the most important of all facts to the practical brewer—i.e., that the air in the fermenting room itself may contain a multitude of those germs which are productive of the most calamitous results. It is, however, possible to keep the air free from these invisible organisms, and there is no doubt that the recorded results are directly due, first, to the purification of the air entering the fermenting-room by passing it over brine, and, secondly, to the rigidly maintained order and cleanliness in the cellars of the Old Carlsberg brewery. Hansen's investigations, therefore, point a moral which cannot be too frequently emphasised.

Saito carried out very comprehensive investigations on the distribution of moulds at different times of the year in many places in Tokio, both in the open and indoors, with the aid of Soja-gelatine (Soja, decoction of onions and cane sugar), confirming by this means the results of earlier investigations already cited.

The zymotechnical analysis of water has been of greater value to the brewing industries than the analysis of air. The germs contained in water which give rise to disease in fermentations and fermentation products are not usually so enfeebled as those in air, and water on many occasions comes into closer touch with the different products during manufacture than does air. The examination of water in reservoirs, and the effect of filtration on the micro-organisms, is of especial practical value.

A few details may be quoted here from the researches of Holm and of the author as examples of the results obtained from this form of investigation in the fermentation industry.

Holm's researches showed that among the various micro-organisms in water the *moulds* are those which develop most quickly in flasks containing wort and beer, and generally also

those which occur in largest numbers in the flasks. *Penicillium glaucum* and *Mucor stolonifer* were found among them.

Next to the moulds come the bacteria when wort is infected with water, whilst if sterilised beer is used, they develop only scantily. The following bacterial forms were found :—*Bacterium aceti*, *Bact. Pastorianum*, a third form which made the beer slimy and ropy; and lastly, several species which imparted a disgusting smell to the wort.

Yeast-like cells were of rare occurrence. Holm did not observe any growth of *Saccharomyces*, although some *Torula* forms and *Mycoderma* occurred.

The number of these germs varied at different times of the year, yet it did not seem to be dependent on the season—the rainfall, the condition of the surface water, and of the air had great influence. Of practical importance was the discovery of strong contaminations injurious to wort and beer, in reservoirs situated near granaries and malt-lofts insufficiently protected against dust. It was also shown that water which had been filtered through charcoal filters contained much larger numbers of wort bacteria than the unfiltered water.

The water analyses made in the author's laboratory during a period of more than twenty years have given the following chief results :—The samples of water in only very few cases were found to contain *Saccharomyces* (culture yeasts or wild yeasts). In one series of analyses *S. anomalous* and *S. membranae faciens* were met with. The bacteria observed by Holm which produced slime formation or imparted a putrid smell to the wort, occurred very frequently. If a pure yeast was infected with such species and used for pitching hopped wort, these bacteria did not usually develop further. Although, however, the bacteria did not develop during the fermentation, a difference was often observable between the condition of this beer and that of beer fermented with pure yeast. Acetic acid bacteria were not infrequently found in the analyses, and were usually able to assert themselves in the flasks, even in competition with rival species. In a few cases the experiments with wort showed a growth, and sometimes even an abundant one, of *Sarcina* forms, which did not occur in the

parallel series of experiments with sterile beer. They rendered the wort turbid, and imparted a peculiar smell to it. Among the moulds the following were the most frequent:—*Aspergillus*, *Mucor stolonifer*, *M. mucedo*, *Oidium lactis* and *Dematiump-like* forms. In the water conduits of the breweries a coherent layer of *Crenothrix* was not infrequently found.

In many cases it has been proved that water received a very considerable contingent of its wort and beer organisms in the reservoirs or conduits, and it may safely be asserted, as the result of many years' experience, that brewery water is most seriously contaminated in the brewery itself.

Biological analyses of natural and artificial ice have shown that in both, organisms can exist capable of developing in wort and beer. *Sarcina*-like bacteria can also be introduced along with ice into these liquids, and may develop freely in them. In artificial ice, the inner snowy layer of the ice-block appears to be particularly rich in micro-organisms.

If large quantities of water are to be analysed, it is of the utmost importance to take due care that real average samples are obtained.

Hansen gives the following method for the zymotechnical analysis of air and water, a method based upon a long series of comparative trials.

The principle underlying it is as follows:—For brewing purposes it is only necessary to know whether the water and the air contain germs capable of developing in wort and beer. This cannot, as was formerly assumed, be ascertained by means of the meat decoction peptone gelatine employed in hygienic air and water analysis. The zymotechnologist has this great advantage over the hygienist, that he is in a position to make direct experiments with the same kind of liquid as that employed in practice—namely, wort. All disease germs that have hitherto been shown with certainty to occur in beer are also capable of developing in wort. Hansen's comparative investigations have proved that the use of gelatines introduces great sources of error. Thus, for instance, in a series of comparative experiments with corresponding samples of water, the following numbers were obtained:—In Koch's nutrient gelatine—100, 222, 1,000, 750, and 1,500 growths obtained from 1 c.c. of water; in wort—

0, 0, 6.6, 3, and 9 growths ; whereas, in beer, none of these water samples gave any growth. In another series, Koch's gelatine gave for 1 c.c. of water 222 growths, wort gelatine 30 ; but none of the flasks containing wort or beer, after infection with the water, showed any development of organisms. Thus, only very few of the great number of germs living in the water developed in wort or beer.

Hansen has further shown that in zymotechnical analyses of water and air, it is a mistake to employ gelatine at the outset, and then to transfer the colonies that have been formed into wort. Thus, he demonstrated by experiment that several of the bacterial germs occurring in atmospheric dust and in water are capable of developing in nutrient gelatine, but not in wort ; whilst several of these species become invigorated to such a degree, after having formed a new growth in the gelatine, that they are then enabled to develop in the less favourable medium, wort. Another, and a still greater, objection to the gelatine method is that several organisms, and just those of importance, do not develop at all when transferred directly to the gelatine in the enfeebled condition in which they generally occur in atmospheric dust and in water.

Temperature naturally plays a much more important part in the development of the germs on gelatine plates than in nutritive liquid ; at a less favourable temperature they will develop with greater difficulty in gelatine, owing to its deficiency in nutrient.

Reference may also be made in this connection to the difficulties that are encountered in determining the number of germs which can develop on gelatine plates. Species of frequent occurrence in water, that tend to liquefy gelatine, generally develop very rapidly, and may encroach so extensively on the space that other species do not succeed in forming colonies at all ; others require so long an incubation before visible colonies are formed that the examination of the plates is often concluded before these growths appear.\*

\* In comparative investigations, as, for instance, the examination of air and water before and after filtration, gelatine plates are usually employed ; the possible sources of error accompanying their use must, therefore, be borne in mind.

Based upon these observations, Hansen devised the following method :—Small quantities of the water, either in its original state or diluted, are added to a series of Freudeneich flasks containing either sterilised wort or beer.\* After incubation at 25° C. for fourteen days the contents of the culture flasks are submitted to examination. If only a part of them show any growth, the rest remaining sterile, it may be assumed with approximate certainty that each of the flasks belonging to the former set has received only a single germ. Information is thus gained concerning the number of germs capable of development existing in an ascertained volume, and the different germs are also under more favourable conditions for their free development. An exact examination will then show to what species these germs belong.

Although wort-cultures give a very small number of growths in this method in comparison with plate-cultures, yet in many cases the number of wort-growth will still be too high, for these growths are able to develop in the flasks undisturbed and without hindrance from other organisms, but when wort is mixed with good culture-yeast in the fermenting vessel, many of these germs will be checked. Further, the flasks which show a formation of mould will have no importance for the brewery itself, but only for the malt-house. In order that the conclusions based on the results should approximate more closely to practical requirements, Hansen proposes the following method of procedure :—The flasks containing a development of yeasts and bacteria are divided into two groups—(1) those in which the growths appear rapidly, and (2) the remainder, in which they make their appearance later; for instance, after five days. Among the latter are those species which develop less readily in wort. To these, then, less importance is attached in forming an opinion as to the nature of the water or air.

The same principle is used by Wichmann, who endeavoured to give a numerical expression for the “harmfulness” of water—that is to say, to express to a definite degree the destructive property of water with regard to wort and beer.

\* In the analyses of air the germs are aspirated into sterilised water, or first into cotton-wool and then transferred to water.

He impregnated a series of Freudenberg flasks, containing sterile, clear wort or beer, with different volumes of water, and noted the day on which a change in the contents of each flask (cloudiness, formation of a skin, fermentation) became visible. The more rapidly decomposition sets in, and the smaller the necessary quantity of water, the more noxious the character of the water. By giving numerical expressions to the time (setting in of decomposition), and to the quantity of water, the figure specifying the destructive capacity will be a product of these two factors. Beer, however, possesses a greater power of resistance than wort, so that in cultivations in the former, the coefficient must be correspondingly reduced.

Lindner added sterile wort to the water, and distributed the mixture by means of a pipette drop by drop into a series of Petri propagating dishes. The growths which developed were counted, and from this the number of germs per c.c. was calculated.

The result of many years' experimenting in the author's laboratory have led to conclusions which are at variance with those stated above. The first growths to appear in the wort flasks are almost always putrefactive bacteria, water bacteria, and the like; just those forms which are of little interest in brewing operations, because they do not develop in the finished product. The special technical character of the analysis is lost if the time taken for the appearance of the bacteria is made the basis on which the character of the water is judged. Moreover, in forming an opinion as to the character of water from the technical standpoint, it is not essential that the quantity of wort-bacteria in a given volume should be estimated. On the contrary, when growth in the flasks has been allowed to continue for some time, it has been shown that in more advanced stages species appear which are known as disease germs, as, for instance, the wild yeast. Thus it is the last stage of the development in the flasks which is of real importance. In making the investigation both the wort and the beer flasks should be infected with small quantities of water. In some flasks the water is added in an undiluted condition; in others it is more or less diluted. Samples are taken from time to time for microscopical, and eventually

for microbiological examination, in order that the micro-organisms which appear may be more closely investigated in regard to their action on wort and beer. For water analyses in connection with distilleries and allied industries, sweet wort is usually employed. This has been found to answer the purpose well, and is easier to obtain clear after sterilising than the mash—*i.e.*, the worts from distilleries and yeast manufactories. If special problems arise, as, for instance, the presence of certain species of micro-organisms, the analytical apparatus should be modified to suit the characteristics of the particular species. Thus, for yeast factories, the appearance of moulds and putrefactive bacteria will be of special importance. Provision for the former can be made by cultivation of the water on the surface of congealed gelatine which has been mixed with a decoction of fruit, and for the latter by employing ordinary plate-cultures consisting of neutral nutrient gelatine. For special demonstration of the *Sarcina* forms in beer a neutral decoction of yeast, with the addition of a small quantity of alcohol, will be suitable. For developing “wild yeast,” wort may be used with an admixture of hydrofluoric or tartaric acid, and so on.

Experiments may also be arranged in connection with other branches of the brewing industry by mixing the liquid which is to be fermented with a certain quantity of the suspected water at different stages of the fermentation, the addition of pure yeast having been previously made. The difficulty of this method when working with small quantities consists, as is well known, in approaching sufficiently near to practical conditions to make it possible to draw direct conclusions. If, as is the case in our own laboratory, it is desired to ascertain what groups or species of organisms occur in the water, then it will be of no consequence if the sample has been delayed in transit.

## CHAPTER III.

## BACTERIA.

BACTERIA occur in every shape, from the smallest specks or spheres to green algæ-like filaments; and they are found in nearly all possible localities, under the most varied conditions. According to their action, a distinction is made between pathogenic, zymogenic, and chromogenic bacteria, or those that produce disease, fermentation, and coloration respectively.

Our first knowledge of these living forms was obtained by placing small quantities of different substances under the microscope, and examining them with high powers. In putrefying meat minute spherical bodies were found, which clearly multiplied by division; in sour milk short rods occurred, and in decomposing vegetable matter large spherical bodies and long fine threads; in the mucus of teeth very fine spiral threads were found. Thus it was convenient provisionally to retain these forms, and to describe them as independent species. Credit is due to Cohn for the first systematic classification of bacteria.

Bacteria always consist of single cells. In their simplest form they occur as spherical bodies (*coccus*, Fig. 16, *a*) of varying size, often so small that they can only just be seen even with the strongest powers, and only give evidence of their existence as organisms during propagation by division. If the *coccus* divides in one plane, *Diplo-* and *Streptococcus* are produced (*b*) (*c*). By division in two planes, the *Micrococcus* (*b*) is obtained, and by division in three directions the *Sarcina* form.\* If the cells assume a cylindrical form we have bacteria (*e*).

\* In the *Sarcina* forms that occur in beer, the division is incomplete in all three directions, but appears to vary, so that an irregular piling up of spherical bacteria takes place, or else a marked displacement of single cells is found. Two spherical bacteria are often found strung together. All such conglomerates of cells are surrounded by a gelatinous envelope, the development of which is dependent upon the nutritive conditions.

which may be of very varied length. A distinction may be drawn between the motile (*bacillus*) and the motionless form (*bacterium*). When the rods are swollen in the middle, and thus form spindle shapes, we have the *Clostridium* form (*f*). If the bacilli are elongated, so as to become more or less thread-like, they are called *Leptothrix* (*g*), which may also occur as pseudo-filaments when several bacteria are grouped lengthwise, or as *Cladothrix*, when they lie close to one another and appear as irregular branching threads. The bacilli and

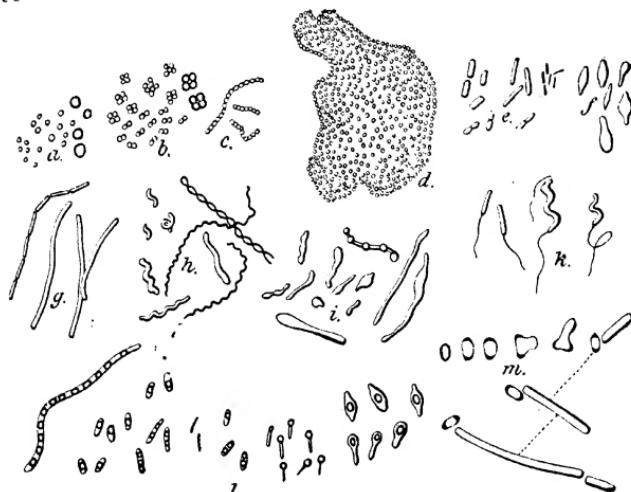


Fig. 16.—Growth-forms of Bacteria (in part schematic)—*a*, cocci; *b*, diplococci and micrococci; *c*, streptococci; *d*, zoogloea; *e*, bacteria; *f*, clostridium; *g*, pseudo-filament, leptothrix, cladothrix; *h*, vibrio, spirillum, spirochete and spirulina; *i*, involution forms; *j*, bacilli and spirilla, with cilia or flagella; *l*, spore-forming bacteria; *m*, germination of the spore (*Bacillus subtilis*).

filaments frequently assume wavy or spiral forms (*h*); when they are only slightly curved, we have the *Vibrio* form; when the spirals are more prominent, the *Spirillum* and *Spirochete* forms; when they intertwine like a plait of hair, the form called *Spirulina* is produced.

The thickness of most bacteria is about  $\frac{1}{1000}$  mm. ( $1\mu$ ); the largest do not exceed 3 to  $4\mu$ . The thickness may, however, vary extraordinarily in one and the same kind, and the same is equally true of the length, because the cells elongate before they subdivide. The limit may be placed at 10 to  $12\mu$ ;

only filaments exceed this. The conditions of nutriment play an important part in determining the shape of any one species.

In all probability bacteria exist of such small dimensions that they cannot be distinguished with existing microscopes. Thus, Roux has shown that, in pleuro-pneumonia of cattle, an organism occurs which develops colonies on the substratum, while it is impossible, even with the strongest power, to see the individual bacteria.

A single species of bacteria may occur in various forms, and this may happen either during the normal growth of the vegetation,\* or in growths on different media. Cases also occur in which the same species may yield different vegetative forms, which by prolonged cultivation under different conditions give rise to so-called varieties. Isolated cells occur in the growth of many bacteria which differ from the rest in that they are irregularly swollen or branched; these have been named involution forms (Fig. 16). In some species such cells represent a diseased growth—possibly a consequence of harmful ingredients in the culture medium—and they occur more particularly in old cultures; in other species, for instance the acetic acid bacteria, they appear, on the contrary, to belong to a certain stage of the vegetative growth.

The bacterial cells contain protoplasm, a homogeneous, feebly refractive substance, which may contain bright little granules. Occasionally one or more clear spaces are found within the cell, which by analogy with the higher plants are regarded as sap-cavities or vacuoles. By staining, Rayman and Kruis have rendered dark round bodies visible in the centre of certain bacteria, which they named cell nuclei. The granules that occur in the plasma are, without doubt, of very varied character, even in one and the same cell. In many bacteria, fat globules have been distinguished with

\* As an excellent example of the regular occurrence of different forms, the common hay bacillus will serve (*B. subtilis*). From the spore a motionless rod develops which subdivides, the new individuals being transformed into swarming cells. These then grow into very long, motionless threads which subdivide, and, in each individual, one spore forms which is finally set free by the breaking down of the surrounding cell wall.

certainty ; in sulphur bacteria, granules of sulphur occur as brightly refractive globules. Other granules occur in plasma, which contain either starch or glycogen, especially before the formation of spores ; they are used up as the spores develop. These granules are coloured either blue or reddish-brown on treatment with iodine.

Surrounding the protoplasmic body we find a **cell-wall** or **membrane**. By treatment with a hygroscopic substance the inner surface of the cell-wall stands out clearly, owing to the contraction of the plasma. An examination by Löffler's method of staining generally shows that its outer layers are swollen up into a gelatinous mass, which becomes quite distinct when large numbers of bacteria are massed together. From a chemical standpoint it must be provisionally assumed that the cell-wall is differently constituted in different species. In some it recalls the cellulose of the higher plants, whilst in others it appears rather to resemble the albuminoids in its properties.

In decomposing liquids, as well as in the slime-fermentations, bacteria are frequently observed, in which the outer layers of the cell-wall have been converted into a viscous condition, so that the mass of cells is embedded in a structureless slime. Such structures are called *Zooglaea*. When the slime has a sharply defined edge, and doubtless a harder consistency, it has been described as a capsule formation, as, for instance, in the "frog-spawn" (*Leuconostoc*) of the sugar factory. Occasionally the outer layers of the cell-wall are surrounded by a sheath of firm consistency, which eventually encloses a large number of independent cells (*Crenothrix*). The enclosed cells multiply inside the sheath until at last they force their way out, and the sheath survives for a time as an empty husk.

At this point we may note the remarkable phenomena which Thaxter, Bauer, Quehl, and others have described. *Myxo-bacteria*—i.e., swarms of organisms occurring particularly on excrement—which multiply by division, exhibit a slow crawling motion, and during their growth secrete a colourless slime, in which they live, and which enables the swarm to hold together. The rods are eventually transformed

into spherical spores, which form small red clusters, or else a number of small rods become enclosed in a common membrane, termed a cyst. With these may be associated the remarkable slime-formation, packed with bacteria (*Bacteroids*), which penetrate through the root-hairs into the nodules of the leguminosæ, and assimilate nitrogen from the atmosphere. Lastly may be mentioned the remarkable "bacteria-bubbles" (*bacteriocysts*) described by Müller-Thurgau, which occur in fruit wines containing tannic acid, especially in perry, and more particularly in and upon the yeast which settles after fermentation. These are zoogloea forms of lactic acid bacteria, which surround themselves with a membrane, and thus resemble the cells of higher plants. Inside this membrane the bacteria are embedded in a clear mobile liquid, and they eventually collect in the lower part of the bubble. These may gradually increase in size. There is a certain resemblance between these and the remarkable forms described by Winogradsky in 1888, the *Amœbacter* (sulphur bacteria), which also occur in cell families, the cells being bound together with threads of plasma, and the whole family moving as a slimy mass (amœbæ) in ever-varying forms. When these reach the resting stage, a thin gelatinous skin separates out, and is slowly transformed into a hard skin enclosing the whole family, which eventually breaks away from its husk.

Many bacteria contain blue, red, yellow, or green **colouring matter**, which may cause intense coloration. In most bacteria of this category the colouring matter is present in solution in the nutritive liquid, whilst the bacteria appear to be colourless. In other cases, on the contrary, the colouring matter is found in the cells, for instance, in the red sulphur-bacteria, where the red colour plays the same part in the nutrition of the bacteria that chlorophyll plays in the higher plants. One of the commonest pigment bacteria is the *Bacillus fluorescens liquefaciens* commonly occurring in water, which yields a greenish-yellow, fluorescent colour, soluble in water.

**Phosphorescent** bacteria are found more particularly in sea water; great numbers occur on dead animals and plants. The phosphorescent phenomenon is connected with aération, for it ceases when air is excluded.

With regard to the **chemical composition** of bacteria, a number of analyses have been published. Before the analyses were made, the growth was thoroughly washed to remove, as far as possible, every trace of the culture medium. They show a content of about 85 per cent. of water, 8 to 14 per cent. of albuminoids, 1 to 4 per cent. of fat and waxy bodies, and about 1 to 2 per cent. of ash (sulphur, phosphorus, chlorine, potash, lime, magnesia, iron, manganese, and silica). Their composition is, however, obviously influenced to a considerable extent by the nutriment. The slime formed by many bacteria is either a compound of a carbo-hydrate and an albuminoid, or a carbo-hydrate alone, as in the case of "frog-spawn" (*Leuconostoc*) and similar species. Bacteria contain a number of enzymes of a more or less pronounced albuminoid character, and this is also the nature of the various poisonous substances which occur in several species.

For **nutrition**, bacteria require carbonaceous and nitrogenous compounds, as well as the inorganic substances found in the ash. The majority of bacteria do not appear to possess the power of building up their organic constituents from inorganic material ; they are dependent upon those organic compounds that have already been built up in animals and plants. The nitrifying bacteria form an exception in that they can directly absorb carbon dioxide from the air, and the bacteria which occur on the nodules of the leguminosæ in like manner absorb nitrogen from the air and assimilate it.

It has been customary to distinguish between **Saprophytes** or organisms of putrescence and **parasites** which feed only upon living animals and plants. A corresponding classification of the bacteria has been attempted in a biological sense. Alfred Fischer divides them into **prototrophic**, **metatrophic**, and **paratrophic** (from the Greek, *trophä*, nourishment).

By **prototrophic** bacteria are meant those, like the nitrifying, the iron and sulphur bacteria, which can take up these substances in an inorganic form. The vast majority of bacteria are **metatrophic** ; they utilise organic compounds of the most varied kind, while they promote putrescence or fermentation. Lastly, the **paratrophic** are parasites ; they do not occur in nature in a free state, but can only grow upon other forms of

life. Nevertheless, it is possible to cultivate them—e.g., in blood serum—at the temperature of the body.

The metatrophic bacteria, which form the vast majority, and are of special interest to the fermentation physiologist, are not equally responsive to the different carbonaceous and nitrogenous food-stuffs. Peptone and amides are good sources of nitrogen. Many bacteria can also utilise ammonium salts and nitrates under given conditions, thus *Bact. aceti* can assimilate ammonia in presence of acetic acid. Similarly, according to Henneberg, certain species of acetic acid bacteria can utilise potassium nitrate and ammonium tartrate as sources of nitrogen, if the culture material contains sufficient dextrose.

The carbohydrates constitute the most important source of carbon. Of the different varieties of sugar, grape sugar forms an excellent food for bacteria.

According to the conditions of nourishment, bacteria may bring about varying decompositions of the substrata.

The different nutritive fluids and gelatines that are used in the culture of bacteria are described in Chap. i., sec. 6.

Pasteur made the important discovery that there are certain bacteria and other micro-organisms which do not require free oxygen, but are capable of effecting active decomposition of the fermenting material, even when oxygen is excluded. He, therefore, distinguished two classes of micro-organisms, aërobic and anaërobic. Whilst the aërobic bacteria breathe in a similar manner to all other organisms, and thereby convert organic substances (non-nitrogenous) into carbon dioxide and water, and bring about similar decompositions with the nitrogenous compounds, the anaërobic bacteria comprise on the contrary those whose life activity is sustained without free oxygen. To this class belong some of the butyric bacteria, as well as the bacteria that ferment cellulose.

Since Pasteur's discovery (1861) numerous bacteria have been investigated in this respect, and it has been proved that there is every possible transitional stage between the obligatory aërobies (amongst which the hay bacillus, *Bac. subtilis*, must be classified) and the obligatory anaërobies. A number of facultative anaërobic species are now known which grow well with access of air, but also develop, to a degree varying with

the species, either in diluted air or even in the absence of oxygen ; examples may be found among the lactic acid bacteria. It is a well-established fact that with one and the same species the demand for oxygen varies according to the other life conditions. The heat-loving (thermophilous) bacteria form a typical example, for they can grow at a high temperature in the presence of air, whilst at a low temperature they grow only in the absence of air. Of the obligatory aërobæs many, when well nourished, can develop in air containing only traces of oxygen.

Whilst a few bacteria are motionless (*e.g.*, lactic acid and, in part also, acetic-acid bacteria), the majority show a capacity for free movement ; such bacteria are commonly met with in decomposing fluids. This motion, which is not to be confounded with the Brownian molecular movement, usually consists of a forward swimming action, together with a rotation round the longer axis. The organs of motion, which only become visible on staining, consist of fine protoplasmic hairs—flagella or cilia—which are connected with the plasma of the cells through holes in the cell-wall. A few species have only a single cilium, attached to one end of the cell ; others have a bunch of cilia at one end, whilst cilia are distributed over the entire surface of others—*e.g.*, the hay bacillus, in certain stages of development, and some of the common putrefying bacteria.

By means of these organs of motion, bacteria are enabled to penetrate to that part of the nutritive fluid which offers the most favourable conditions for existence. Thus, Engelmann has proved that aërobæs move to the stratum of liquid which is richest in oxygen, whilst the anaërobæs move in a contrary direction. Similarly, Pfeffer has shown that bacteria move to these parts of a fluid that contain nutriment of suitable concentration. The rate of motion is conditioned by temperature ; thus, *Bac. subtilis* moves more rapidly at 37° C. than at 20° C., whilst other bacteria cease to move at the former temperature.

The propagation of bacteria takes place by division. It has been observed in detail in the larger species. The cells expand, fine transverse lines appear, which gradually increase in thickness and split into two leaflets ; after this the organism separates into smaller rods, which sometimes remain united,

sometimes become detached (Fig. 24, *A*). Long before a trace of these transverse walls can be observed, staining will show that the organism consists of a series of segments, each of which corresponds to a subsequent individual. The newly formed segment cells are all in the same plane. A division in either one, two or three planes has been observed in certain cocci.

In the case of many bacteria, formation of **spores** takes place in the following manner (Figs. 16, *l*, *m*; 24, *B*). The plasma in the cell becomes darker, and often distinctly granular; a small body subsequently appears—frequently at one end of the cell—strongly refractive to light, which quickly increases in girth, and is surrounded by a membrane. Meanwhile, by far the greater portion of the remaining plasma of the cell disappears, being used up in the formation of the spore. This is seen enclosed in a clear liquid which gradually disappears, and finally the cell-wall shrivels up, and only remains as a withered appendage to the ripe, egg-shaped spore. In many cases a swelling takes place in the mother-cell during spore-formation (Figs. 16, *l*; 24, *B*). Before spore-formation begins, the cells of many, especially anaërobic species, are coloured blue with iodine like starch-granulose. Probably at this stage the cells store up reserve food material. Usually only a single spore is formed in a cell.

One cause for spore-formation is that during vegetative growth the products of its own activity—acids, alkalies, etc.—accumulate in the nutritive substratum, and as a consequence, further vegetative growth is checked. The exhaustion of the nutritive medium may produce the same effect. Spore-formation demands a suitable temperature, and a certain quantity of moisture. The membrane of the spore is very strongly developed, and is frequently surrounded by a gelatinous envelope. The contents are strongly refractive, and contain but little moisture. Spores are of value in enabling the species to survive when conditions occur that are unfavourable to vegetative life. They possess quite an extraordinary power of resistance to harmful influences. The membrane cannot be easily moistened or penetrated by water, and the great durability of spores is especially due to the fact that their plasma contains little or no moisture. Thus.

according to Flügge, species occur amongst the peptonising bacteria of milk, the spores of which will withstand boiling for four hours. Spores of hay bacillus will also withstand boiling for hours. Spores can usually stand dry heat better than boiling in steam or water. On the other hand, many spores show special resistance to heating in milk, and the same is true of neutral or feebly alkaline liquids, whereas acid liquids are unfavourable to their existence. Spores are generally difficult to stain. On the other hand, colouring matters once taken up by spores are retained better than by vegetative cells ; and after bleaching, therefore, coloured spores become visible in a colourless cell.

As soon as favourable conditions of nutriment and temperature recur, spores germinate. They first swell up by absorption of water, and the contents lose their strong refractive power. A bacterium then grows out from the spore ; the wall of the latter is sometimes seen to burst or to unfold into two valves (Figs. 16, 24). The full-grown rod then multiplies in the usual manner. Spores may maintain their germinative power through a long period, sometimes for many years.

In addition to the endosporic bacteria just mentioned, "arthrosporic" bacteria were formerly described which do not form spores in the interior of the cell, but in which it was believed that members split off from vegetative cells form the starting point of fresh vegetative generations. A microscopically discernible difference between the "arthrospores" and other cells, however, occurs only in a few cases, in that the walls of the latter thicken (Chlamydospores). Perhaps by continued investigation endogenous spores will be found in all such species.

The gonidia which occur in *Crenothrix* constitute a special kind of cell ; in the case of some bacteria, as, for instance, *Cladotrichix*, they are motile. These cells are organs of propagation.

**Temperature** plays an important part in the life processes of bacteria. We distinguish the minimum, optimum, and maximum temperature at which life can exist. These three cardinal points differ, not only for each species, but also for the individual functions of each, such as its rate of growth and its fermentative activity.

Many bacteria are very resistant to low temperatures. J. Forster, B. Fischer, Miquel, and others have shown that bacteria exist which multiply rapidly at the freezing point. Certain species are not killed by exposure to a temperature of  $-70^{\circ}$  C.,  $-110^{\circ}$  C., or even to the extreme temperatures of  $-213^{\circ}$  C. and  $-252^{\circ}$  C. (Frisch, Pictet and Young, Macfadyen, and Rowland). In contrast to these, a number of *thermophilous* bacteria have been discovered. Miquel has described *Bacillus thermophilus*, which multiplies readily at  $70^{\circ}$  C., whilst its development is arrested at  $42^{\circ}$  C. Other species will only germinate above  $60^{\circ}$  C. In the excrement of animals many species of frequent occurrence continue to grow at  $25^{\circ}$  C., whilst their growth is inhibited at about  $39^{\circ}$  C. (*L. Rabino-witsch*). The lactic-acid bacteria and certain organisms occurring in molasses, in the fermentation of tobacco, and in the spontaneous heating of hay, belong to the *thermophilous* species. The bacteria occurring in hay have been examined in detail by Miehe. F. Cohn has also proved that the cause of the spontaneous heating of moist cotton waste is the presence of a micrococcus belonging to this group. It has already been stated that spores will stand a considerably higher temperature than vegetative cells. It is, therefore, obvious that only high temperatures can be used for disinfection.

With regard to the germicidal action of light, Downes and Blunt found, as early as 1877-1878, that direct sunshine powerfully restricts their growth, and that the most active rays of light are the strongly refractive blue and violet rays, well known to possess powerful photo-chemical properties. On the other hand, red and orange rays are less active, and heat rays which accompany the light rays possess no activity.

H. Buchner and S. Bang, amongst recent workers, have studied the action of light upon bacteria. Buchner records that sunlight plays a part in the spontaneous purification of rivers by bacteria. It is assumed that the effect of direct sunlight on bacteria is not entirely due to the action on the cells, but also to the alteration brought about in the substratum, whereby it becomes less suited for nutrition. For example, the formation of hydrogen peroxide in nutritive agar by exposure to sunlight has been demonstrated. A few quite

exceptional bacteria are known which appear to thrive in bright light. This is true of the purple bacteria, which, like green plants, assimilate carbon in presence of light.

Bacteria are capable of living at considerable depths. Russel found bacteria alive at a depth of 1,100 metres. They are, therefore, capable of withstanding a pressure of over 100 atmospheres, and certain putrefactive bacteria have survived a still higher pressure.

With regard to the action of antiseptics on bacteria, the rule has already been laid down, in the section on sterilisation, that the higher the temperature the more easily they are killed. But, with regard to the restrictive action of antiseptics, it is usually weakest at the optimum temperature, and stronger at both higher and lower temperatures. Very dilute solutions of an antiseptic may encourage the growth of bacteria.

Various species react differently to the same concentration of a reagent, and the action depends to a great extent, with any given species, upon the state of nourishment of the cells. Spores are much more resistant than vegetative cells. It has proved possible to propagate bacteria in the presence of successively increased quantities of an antiseptic, but the characters so obtained prove not to be fixed, but disappear as soon as the culture is prepared in a substratum free from poison.

Bacteria and other micro-organisms when subjected to mechanical vibration behave very variously. Horvath proved that gentle vibration has no action on their growth, whereas violent shaking hinders or entirely inhibits it. Meltzer arrived at the conclusion, after prolonged experiments with liquid cultures, that gentle vibration promotes the multiplication of micro-organisms; with a given degree of motion the rate of germination of the species is at the maximum, whilst any stronger vibration restricts it. The optimum and maximum differ for each species. According to Appel, cultures of bacteria on solid substrata behave the same whether they are shaken or not.

Attempts have been made ever since the discovery of bacteria to define this large group of organisms, and to classify the various species in one system, like other sections of the vegetable kingdom.

With the exception of a small number of doubtful forms, amongst which *Crenothrix* may be named, the bacteria form a fairly uniform group, exhibiting the same simple structure and the same method of propagation throughout. It is an interesting fact that amongst the lowest green plants, the algae, there is a group which exhibits the same construction and the same method of propagation as the bacteria, so that the lowest green plants connect up with the lowest fungi. It has already been mentioned that green plants possess the power of absorbing and assimilating the carbon dioxide of the air, owing to a special constituent of the cells, chlorophyll. This power is not possessed by fungi, and the lowest group of algae has been classified, therefore, under the name of *Schizo-algae*, in contrast to the *Schizo-fungi*. It is not only a physiological difference that distinguishes the two groups—that could not be used as the basis for a systematic classification—but rather that the structure of the cell contents is entirely different, since the *Schizo-algae* contain grains of chlorophyll. On the other hand, the *Schizo-algae* have not the power possessed by bacteria of forming endogenous spores, but the method of division exhibited by *Crenothrix* (see Fig. 31) frequently occurs in the algae. To illustrate how difficult it is to define strict limits in nature, it may be stated that there are undoubtedly bacteria which contain green colouring matter, and yet others that can assimilate atmospheric carbon dioxide.

Most people are agreed that bacteria are to be classed amongst the fungi, although, in their method of propagation, they stand nearer to the algae. There are, however, fungi which show the same kind of cell division as bacteria, and even the remarkable formation of endospores also occurs amongst many fungi, especially amongst those that will occupy us next, the *Saccharomycetes*.

We have seen that bacteria have points of contact, both with the lowest forms of algae and also with the lowest forms of fungi. It must also be noted that in so far as certain bacteria move about with the help of cilia, a similar relationship exists between them and the lowest group of the animal kingdom (the flagellata), which again have other characters in common with bacteria.

On account of their simple structure, the attempt to form a true system of bacteria is surrounded with great difficulties. It must, indeed, be based upon morphological characters. Thus it came about that for a long time it was generally held (Billroth, Nägeli) that no single species can possibly endure, for it may pass freely and without limit into any other species, and it was further assumed that a so-called species can react in various ways upon its substratum, so that physiologically no hard and fast lines can be drawn. In both directions the hypothesis went far beyond the facts, and it must be considered to have been a great advance when Cohn, in 1875, first published his system of bacteria. His system must now be regarded as out-of-date, since Zopf, de Bary, van Tieghem, Hueppe, and many others have established new divisions, which correspond more closely to the natural boundaries. Nowadays general use is made of the schemes established by A. Fischer and Migula, and we must limit ourselves to describing the main lines of Migula's system. He recognises two orders, the *Eubacteria* and *Thiobacteria*, the latter distinguished by containing sulphur, and being either colourless or coloured pink, red, or violet by bacterio-purpurin.

#### I. Order—EUBACTERIA.

##### 1. Family—*Coccaceæ* (Zopf) Mig.

Cells in free condition completely spherical, in state of division somewhat oval.

(1) Genus *Streptococcus*, Billroth. Cells motionless, round, division only in one plane, occurring singly, in pairs, or in chains like strings of pearls.

(2) Genus *Micrococcus* (Hall), Cohn. The cells divide in two planes, and, after subdividing, combine to form plate-like layers. No cilia.

(3) Genus *Sarcina*, Goods. The cells divide in all three planes, and after subdivision mass together in the form of bound packets. No cilia.

(4) Genus *Planococcus*, n.d. The cells divide in two planes like *Micrococcus*, but possess cilia.

(5) Genus *Planosarcina*, n.d. The cells divide like *Sarcina* in three planes, but possess cilia.

##### 2. Family—*Bacteriaceæ*.

The cells are cylindrical and vary in length; they are straight, and never twisted like a screw; division takes place only in one plane after the rods have expanded in length.

(1) Genus *Bacterium*. Cells without cilia, often forming endospores.

(2) Genus *Bacillus*. Cells covered entirely with cilia; often forming endospores.

(3) Genus *Pseudomonas*. Cells with polar cilia only; seldom form endospores.

### 3. Family—*Spirillaceæ*.

Cells twisted spirally, or forming part of a spiral-like curve. Division only in one plane after cells have expanded in length.

(1) Genus *Spirosoma*, n.d. Cells without cilia; rigid.

(2) Genus *Microspira*. Cells rigid, with one, and, less frequently, with two or three polar and undulating cilia.

(3) Genus *Spirillum*. Cells rigid, with a bushy formation of polar cilia, shaped in semi-circular curves.

(4) Genus *Spirochæte*. Cells in serpentine curves; cilia unknown.

### 4. Family—*Chlamydobacteriaceæ*.

Cells cylindrically arranged in threads surrounded by a sheath. Propagation through motile or motionless gonidia, which project from the vegetative cells, and grow into new threads without undergoing any resting stage.

(1) Genus *Chlamydothrix*, n.d. Cells cylindrical, motionless, without branches. Grouped as threads surrounded by thick or thin sheaths, with nothing to distinguish one end from the other.

(2) Genus *Crenothrix*, Cohn. Thread-forming bacteria without branches, differing at the ends; stationary. Thick sheaths often impregnated with ochre. Cells at first divide in one plane, and later in two or three planes. The products of division are rounded off, and grow into gonidia.

(3) Genus *Phragmidiothrix*, Engler. Cells at first assembled in unbranched threads, which divide in three planes, and so produce a strand of cells. At a later stage the single cells penetrate through the fine close sheath, and give rise to branching.

(4) Genus *Sphaerotilus* (incl. *Cladothrix*). Cells cylindrically enclosed in sheaths, forming asymmetric branched threads without distinction between the ends. Propagation through gonidia, which swarm through sheath, and settle on surrounding objects, developing immediately into fresh threads. The gonidia possess bushy polar cilia.

## II. Order—THIOBACTERIA.

### 1. Family—*Beggiatoaceæ*.

Bacteria forming threads, containing no bacterio-purpurin.

(1) Genus *Thiothrix*, Winogradsky. Motionless threads clustered together, and surrounded by a fine sheath. Division takes place in one plane. At the end of the thread rod-like gonidia form, having a crawling movement.

(2) Genus *Beggiatoa*, Trevisan. Threads without sheath formed of flat cells, crawling like the *Oscillaria*, and rotating round their axis in a free condition. Gonidia unknown.

2. Family—*Rhodobacteriaceæ*.

Contents of the cell coloured pink, red, or violet by bacterio-purpurin; cell containing granules of sulphur.

(1) Sub-family—*Thiocapsaceæ*.

Cells grouped in families; division in two or three planes.

(1) Genus *Thiocystis*, Winogradsky. Small families closely packed, surrounded by one or more gelatinous cysts; motile at all stages of existence.

(2) Genus *Thiocapsa*, Winogradsky. Flat spreading colonies on substratum, consisting of spherical cells loosely embedded in one mass of jelly; motionless.

(3) Genus *Thiosarcina*, Winogradsky. Family grouped in a packet form, incapable of movement. Corresponding with genus *Sarcina* in the *Eubacteria*.

(2) Sub-family—*Lamprocystaceæ*.

Cells combined in families. Division of cells first in three, and afterwards in two, planes.

(1) Genus *Lamprocystis*, Schröter. Families at first solid, then forming hollow spherical mesh, and finally separating into small motile groups.

(3) Sub-family—*Thiopediaceæ*.

Cells grouped in families. Division in two planes.

(1) Genus *Thiopedia*, Winogradsky. Families forming plates consisting of motile cells arranged rectangularly.

(4) Sub-family—*Amoebobacteraceæ*.

Cells grouped in families; division in one plane.

(1) Genus *Amoebobacter*, Winogradsky. Cells grouped in families; division in one plane. Families moving like *Amaba*. Cells wrapped in threads of plasma.

(2) Genus *Thiotece*, Winogradsky. Family with thick gelatinous cyst. Cells loosely enclosed in a common jelly; always motile.

(3) Genus *Thiodictyon*, Winogradsky. Families consisting of small rods, the ends of which are bound together to form a mesh.

(4) Genus *Thiopolycoccus*, Winogradsky. Families solid, motionless, consisting of small densely packed cells.

(5) Sub-family—*Chromatiaceæ*.

Cells free and motile in some stages.

(1) Genus *Chromatium*, Perty. Cells ellipto-cylindrical or elliptical, comparatively thick.

(2) Genus *Rhabdochromatium*, Winogradsky. Cells free, spear or spindle-shaped. Motile at certain stages, with polar cilia.

(3) Genus *Thiospirillum*. Cells free, motile, spiral-shaped. Always motile.

This system provides a survey of the great groups of bacteria, classed together according to their form, structure, and the course of their development.

As soon, however, as a place has been found in the system for any pure species, it is necessary to distinguish it by a detailed and thorough investigation, and to give such an exact description that it can be clearly defined in relation to other species, and may be recognised again if new pure cultures were prepared from the impure material. After the far-reaching work of the last few years, and the thorough exploration to which the field has been subjected, it is possible to give such a description of many species of bacteria, notwithstanding the difficulties due to the great tendency of these plants to exhibit variations. In such a description of a species, it is necessary, owing to the poverty of these micro-organisms in definite shape, to take into consideration many other points. Cohn established the classification of bacteria already mentioned by means of their physiological properties, dividing them into *pathogenic*, *zymogenic*, and *chromogenic* bacteria. Of these, the second group is of importance to us, and the following examples embody typical species of those bacteria that are distinguished either by their special enzymes and fermentative products—acetic acid, lactic acid, etc.—or by their action upon fermenting liquids.

### 1. Acetic Acid Bacteria.

The acetic fermentation is a process of oxidation. By the activity of the bacteria in presence of oxygen, the alcohol in the liquor is oxidised to aldehyde and water, and then the aldehyde is further oxidised to form acetic acid. It is a process differing greatly from those classed as fermentations, and has a certain similarity with the process of breathing. The entire content of alcohol undergoes change without the production of by-products; the bacteria can, however, bring about the combustion of acetic acid to form carbon dioxide and water. It is, therefore, of importance to interrupt the process as soon as acetic acid is formed, if the full yield is to be obtained.

Persoon, as early as 1822, was acquainted with the vegetable character of the film which forms on the surface of liquids undergoing acetic fermentation, and he named the film *Mycoderma*.

In 1837-38 the view was also expressed by Turpin and Kützing that the acetic acid fermentation is caused by a micro-organism, which Kützing described and delineated under the name of *Ulvina aceti*. Starting from this, Pasteur, first in his treatise of 1864 and subsequently in his work, *Études sur le vinaigre*, in 1868, furnished experimental proof of the correctness of this view. He sowed a trace of the film on a mixture of wine and wine vinegar, and thus obtained a stronger development of acetic acid than was possible by allowing the liquid to undergo spontaneous fermentation, and on this he based a process for manufacturing vinegar. He assumed that the acetic fermentation was caused by a single species of micro-organism, which he called *Mycoderma aceti*. His method consists in giving a large surface to the liquid employed—two parts of bright wine to one of wine-vinegar—and then sowing on the surface of the mixture a young film consisting of “mother of vinegar.” When the temperature, the composition of the liquid, and all other conditions are favourable, the formation of acetic acid will proceed more quickly than in the older Orléans process. It is claimed that the installation is cheaper and the loss of alcohol scarcely greater than in the latter process. As early as 1879, E. C. Hansen discovered that at least two distinct species are concealed under the name of *Mycoderma aceti*, which now go by the names of *Bacterium aceti* and *Bact. Pasteurianum*; and now a whole series of species are distinguished. To obtain the best results in this branch of industry, it is again necessary to start with an absolutely pure culture of a methodically selected species. The old Orléans process still prevails in France. In this method the wine which is to be converted into vinegar is placed in casks, half-filled, at about 20° C., to which air has moderately free access. The formation of acetic acid, as in Pasteur’s process, takes place in consequence of the liquid being gradually covered with a film consisting of “mother of vinegar.” In other countries the “quick vinegar process” is employed, in which “the goods” (diluted spirit mixed with vinegar) come into intimate contact with air. To allow free access of air, the liquid is broken up into small drops and distributed over a large surface

(beechwood shavings). The species occurring in this process have been the subject of a far-reaching investigation by Henneberg.

Whilst Pasteur does not explicitly maintain in his memoir that the oxidation of alcohol to acetic acid brought about by bacteria is a purely physiological process, Adolf Mayer expressed this opinion, the correctness of which he confirmed by proving that the vinegar film exercises its greatest activity at 35° C., and that it ceases to react at 40° C., and further that the film cannot react on more than 14 per cent. alcohol. The purely chemical action of platinum black on alcohol presents a contrast, for it is able to react at high temperatures and with higher concentrations.

Pasteur showed that the acetic acid generated by the oxidation of alcohol is transformed, if the oxidation is continued, into carbon dioxide and water. This has recently been confirmed by Adrian J. Brown.

A few species are able to bring about other decompositions, owing to their strong oxidising power—*e.g.*, the formation of butyric acid from butyl alcohol, gluconic acid from glucose—a few, again, have the power of inverting sugar (*Bact. aceti* and *Bact. xylinum*).

An important advance was made in our knowledge of acetic bacteria when Buchner and Meisenheimer, as well as Herzog, proved that this remarkable fermentation is brought about by the activity of an enzyme. The cells may be killed with acetone, and then treated in the same way as the alcohol yeasts (see Chap. v.), and it can then be shown that, after evaporating the liquid, the residue can bring about the acetic fermentation, although it contains no living cells. By this discovery the real nature of the fermentation becomes clear. Like the alcoholic fermentation, it is caused by an enzyme, which may react independently of the living cell that brought it into existence.

These bacteria grow vigorously in many nutritive fluids—*e.g.*, in dextrose solution with peptone and salts. The presence of alcohol is not an essential condition of their existence, and, indeed, more than 4 per cent. of alcohol acts restrictively on their growth.

Hansen's researches are among the first which proved that a definite fermentation is not induced by one species of bacterium only, but by several ; these researches also furnish some of the earliest experimental evidence of the fact that one and the same species can occur in very different shapes ; the correctness of his results was later confirmed by Zopf, de Bary, and A. J. Brown. By means of his staining experiments with *Bacterium (Mycoderma) aceti* (1879), he discovered that at least two distinct species are hidden under this name, of which the one, like most other bacteria, is stained yellow by iodine, whilst the other assumes a blue coloration with the same reagent. For the former he retained the old name *Bact. aceti*, whilst the one stained blue he named after Pasteur—*Bact. Pasteurianum*. The film formations on wort and beer, and likewise the growths on wort-gelatine, give a fine blue colour with tincture of iodine, or iodine dissolved in a solution of potassium iodide, whilst the growths which develop on yeast-water and on broth with peptone and gelatine are coloured yellow ; even very old films on beer show a yellow reaction. It is the slime formation secreted from the cell-wall that is coloured blue. At a later period Hansen discovered a third species.

These three species are characterised as follows :—*Bacterium aceti* (Hansen) (Fig. 17) forms a slimy smooth film on “double beer” (top-fermentation beer, rich in extract, containing 1 per cent. of alcohol), at a temperature of 34° C., and in the course of 24 hours. The slime is not coloured by iodine. The cells of this film consist of rod-bacteria, hour-glass-shaped, and arranged in chains ; occasionally longer rods and threads occur, with or without swellings. At 40°-40.5° C. long thin threads develop. In plate-cultures with wort-gelatine at 25° C. these bacteria form colonies with sharply defined edges, or, more rarely, stellate colonies, which appear grey by reflected light, bluish by transmitted light ; they mainly consist of single rod-bacteria. In peptone-gelatine broth the colonies are surrounded by milky zones, separated from them by

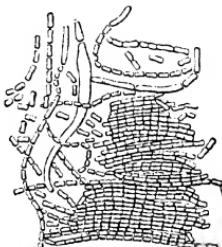


Fig. 17.—*Bacterium aceti*  
(after Hansen.)

clear zones; they may later become iridescent. On sowing drops on wort-gelatine, flat, spreading, rosette-shaped colonies are formed at 25° C. in the course of 18 days. In "double beer" the temperature maximum for growth is 42° C., the minimum 4°-5° C.

This species is of common occurrence both in high- and low-fermentation beers.

*Bacterium Pasteurianum* (Hansen) (Fig. 18) forms a dry film on "double beer" at 34° C., which soon becomes wrinkled and pleated. In young, vigorous films on beer or wort, at favourable temperatures, the slime surrounding the cells is coloured blue by iodine. The cells of the film form long chains, and are, on the average, larger, especially thicker.

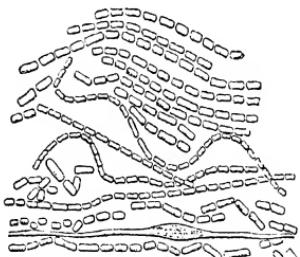


Fig. 18.—*Bacterium Pasteurianum*  
(after Hansen).



Fig. 19.—*Bacterium Kützingianum*  
(after Hansen).

than in the previous species. The thread-like form at 40°-40.5° C. is also a little thicker than that of *Bact. aceti*. In plate-cultures, with wort-gelatine at 25° C., the colonies resemble those of the previous species, but are a little smaller, and consist chiefly of chains. In peptone-gelatine broth the colonies are similar to the previous species. On sowing drops on wort-gelatine wrinkled colonies develop at 25° C. in the course of 18 days, which are slightly raised, and present a sharp outline or one slightly jagged. In "double beer" the maximum temperature for growth is 42° C., minimum 5°-6° C.

This species is more frequently met with in high- than in low-fermentation breweries.

*Bacterium Kützingianum* (Hansen) (Fig. 19) forms a dry

film, on "double beer" at 34° C., which creeps up the side of the flask. The slime is coloured blue under the same conditions as *Bact. Pasteurianum*. The film consists of small rod-bacteria, which are most frequently single or connected in pairs, and seldom form chains. The thread form at 40°-40.5° C. presents almost the same appearance as that of *Bact. Pasteurianum*. In plate-cultures with wort-gelatine at 25° C. the colonies are analogous to those of the previous species. They consist almost exclusively of small, single rod-bacteria. In peptone-gelatine broth the colonies resemble those of the two previous species. On sowing drops on wort-gelatine at 25° C., colonies develop in the course of 18 days resembling those of *Bact. Pasteurianum*, but with a smooth surface without wrinkles. On "double beer" gelatine these colonies are slimy, whilst in the two previous species they have a dry surface.

In "double beer" the temperature maximum of the growth is 42° C., minimum 6°-7° C.

This species was discovered in "double beer."

Hansen's thorough investigation of acetic acid bacteria has assumed great importance in the general biology and morphology of bacteria, owing to the light thrown on one of the factors causing multiplicity of bacterial forms.

Each single species of the acetic bacteria examined by Hansen occurs in three essentially different forms dependent on temperature—chains, consisting of short rods, longs threads, and swollen forms. If sown on "double beer," which is very favourable to their growth, the various species give a growth consisting of chains at all temperatures from 5°-34° C., which develops well, notably at 34° C. If a bit of this young film is transferred to fresh nutritive liquid at 40°-40.5° C., the cells grow into long threads in a few hours (Fig. 20). In some species these threads can attain a length of 500  $\mu$ \* and more, whilst the links of the chain measure only 2 to 3  $\mu$ . If this growth of long threads is then placed at a temperature of 34° C. a transformation into the chain form again takes place. Whilst developing at this temperature, the long threads increase, not only in length, but also in thickness, and

\* 1  $\mu$  = 0.001 millimetre.

that often very considerably. Thus an endless variety of polymorphous swollen forms are produced (Fig. 21). It is not till then that the threads are divided into small links, giving rise to typical chains. Only the thickest parts of the swollen threads remain undivided, and are at last dissolved. Thus the

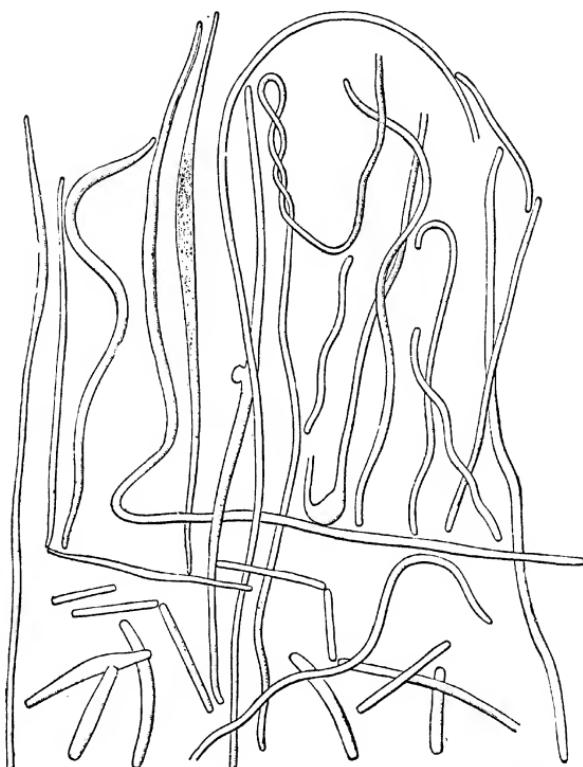


Fig. 20.—*Bacterium Pasteurianum*.—The thread form after cultivation for 24 hours on “double beer” at 40°-40.5° C. (after Hansen).

swollen forms play a regular part in the cycle of changes. This cycle furnishes a striking example of the effect of temperature in determining the form assumed by bacteria.

The species *Bacterium aceti* and *Bacterium Pasteurianum* differ, according to Lafar, both chemically and chemico-

physiologically. In sterilised beer they give different fermentation reactions. At higher temperatures *Bact. Pasteurianum* acquires a higher acidifying power than *Bact. aceti*; on the other hand, *Bact. aceti* is able to carry on a vigorous fermentation at  $1^{\circ}\text{--}4^{\circ}\text{--}5^{\circ}$  C., whilst at this temperature *Bact. Pasteurianum*



Fig. 21.—*Bacterium Pasteurianum*.—Transformation of the thread-forms and chains after cultivation in “double beer” at about  $34^{\circ}$  C. (after Hansen).

forms no appreciable amount of acetic acid. At  $33^{\circ}\text{--}34^{\circ}$  C. *Bact. Pasteurianum* reaches the maximum of acetic acid formation—3.3 per cent. by weight in seven days—after which the formation slowly diminishes, and finally ceases. After the maximum of acid formation has been reached,

irregularly swollen cells make their appearance in the growth, which under the existing nutritive conditions may probably be considered as diseased or degenerate forms (involution forms). More thorough investigation into this question is to be desired. The forms noted by Hansen in the cycle described have quite a different physiological significance, for they are then developing freely, and thus preparing the growth for the formation of new cells.

*Bacterium xylinum* is essentially different from these three species. It was described by Adrian J. Brown in 1886, who examined it especially from a chemical point of view. It forms a film, the slime of which becomes cartilaginous and tough like leather. The growth, consisting of motionless rods resembling *Bact. aceti*, gradually fills up the whole liquid. This species is essentially different from the three first described in another respect—the slimy envelope shows the cellulose reaction, which is not the case with the slime of Hansen's three species. According to Emmerling the slimy sheath contains an albuminoid substance resembling chitin.

According to an investigation undertaken in the author's laboratory, this species occurs in vinegar factories in many countries in a vigorous state of development. According to Henneberg, it may react unfavourably on the aroma of vinegar, and by forming slime may arrest the quick vinegar process.

The sorbose bacteria investigated by Bertrand, which cause the conversion of sorbite, from the juice of mountain ash berries (*Sorbus aucuparia*), into sorbose, is identical with *Bact. xylinum*.

Zeidler found an acetic acid organism in lager beer, *Thermobacterium* or *Bacillus zeidleri*, which occurs as short motile cells and involution forms. When a given quantity of acid has been formed in the liquid, the movement of the cells ceases. If the growth is sown on hopped wort, a cloudy turbidity forms on the surface; the whole liquid gradually becomes turbid, and acquires a yellowish-brown colour. Small protuberances form on the surface of the liquid, which soon sink, and thus a loose brownish deposit is produced. The species does not appear to be dangerous in the brewery.

*Bacterium oxydans*, a species with motile cells, was de-

scribed by Henneberg. The writer discovered it on low-fermentation beer which had been standing in vessels at a temperature of 25°-27° C. It forms roundish colonies on gelatine, which later assume irregular shapes with curious ramifications. On sterilised beer it forms a delicate film, consisting of separate prominences creeping up the sides of the vessel. In its younger stages the film consists of pairs of cells; later, of long chains. Beer is rendered turbid by this species. In its younger stages, motile cells have been observed. At a temperature of 36° C. the growth on beer consists almost exclusively of long, uniform threads. This species also shows the irregular, swollen forms, as, for instance, on beer at 26° C. The cells are not coloured blue by iodine. The optimum temperature for the growth lies between 18° and 21° C. The upper limit of temperature for the formation of motile cells was found to be 37°-40° C. (or 44° when rapidly heated). The temperature at which the vegetation is destroyed lies between 55° and 60° C. for moist heat, and between 97° and 100° C. for dry heat. The oxidation of alcohol into acetic acid has its optimum between 27° and 23° C.

This species oxidises many different kinds of carbon compounds.

Henneberg has recently described the following species or varieties:—*Bact. acetigenum*, which occurs in the quick vinegar vats, forms small rounded swarming cells, which are not grouped in chains. At a later stage swollen cells may appear. The species forms a thin, matted, and very tough film, which finally sinks to the bottom in isolated patches, giving room for a new film formation. By treatment with iodine and sulphuric acid a blue coloration may take place. The acetic acid produced by this species is very aromatic, owing to the formation of acetic ether. The species has its optimum at 33° C.

*Bact. acetosum*, which is found in high-fermentation beer, forms long chains, and, at the same time, irregular shapes. The film is solid, dry, and after a time wrinkled; the liquid is clear. The optimum for this species is about 28° C.

*Bact. industrium* occurs as short swarming cells without chain formation or irregular shapes. On gelatine it forms greyish-

white slimy colonies, and in liquids a thick slimy film and a solid ring attached to the side of the vessel. When shaken, the film separates in flecks. The liquid is rendered turbid. The optimum on wort-agar is 23° C. The upper limit of temperature for motile forms is about 45° C. The species oxidises a large number of compounds. The vinegar produced contains much aldehyde.

*Bact. ascendens*, which is found in wine and wine-vinegar, likewise consists principally of single cells or pairs of cells, but also forms chains. On grape-sugar gelatine and grape-sugar agar the colonies are surrounded by a white halo. In liquids the species forms a very delicate uniform film, which creeps to an extraordinary height up the sides of the vessel. The film is easily broken up and forms a flocculent deposit, and the liquid is rendered turbid. On wort agar the optimum is 31° C. This species is only capable of oxidising a minute amount of material. The vinegar produced is distinguished by its odour of acetic ether. In old cultures the vinegar has a very pungent smell.

Amongst sub-species, or varieties which occur in the quick vinegar process, Henneberg has isolated the following :—

*Bact. Schützenbachi*, which occurs as round, oval, or longish cells, often also sickle-shaped or in irregularly bent and inflated forms, sometimes single and sometimes in chains. On wort gelatine it forms round, clear, glistening colonies with a yellowish-brown centre. On beer-gelatine the old colonies have a whitish, granulated surface. The very thin film that forms on liquids easily sinks to the bottom as a powder. The optimum appears to lie between 25° and 27.5° C.

*Bact. curvum* has rounded, longish, oval, or elongated cells, with either rounded or pointed ends. The more or less bent cells are especially characteristic; it also forms chains. On wort-gelatine the colonies are transparent and rounded, with a raised edge and projecting centre, and frequently have a whitish dry appearance. The film forming on liquids easily falls to the bottom. The optimum lies between 25° and 30° C.

*Bact. orleanense*. The cells vary in shape from spheres to pronounced rod-like forms with all possible transitions. The rods are straight or bent, single or linked in chains, and swollen cells also occur. On wort-gelatine irregular whitish colonies form. On beer-gelatine the older colonies are reddish in colour with a moist glistening surface. The film on liquids adheres firmly, and the liquid, therefore, remains clear. The optimum is at 30° C., and later between 20° and 30° C. The species may be used either for the quick vinegar or the wine-vinegar process.

The same author has described the following special wine-vinegar bacteria (Orleans process).

*Bact. xylinoides* occurs both in the form of fully rounded and of short or long rods ; they may be straight, bent, or irregular, and sometimes swollen, single, or in chains. On wort-gelatine the colonies appear like drops of water, and often exhibit a light brownish nucleus. On beer-gelatine they have a moist glistening, pale brownish surface. The film formation on liquids differs greatly. On sugar-yeast-water and on beer it is thick and tough, like *Bact. xylinum* ; on other liquids it may exhibit every transition from a thin, dry, or smooth, to a thick tough covering. The thick films show the cellulose reaction on treatment with iodine and sulphuric acid, but the thin do not. The optimum for agar-cultures is at 28° C., and later at 20°-23° C. In wine-vinegar mash the optimum lies nearer to 24° than to 28°. The species is found widely distributed throughout wine-vinegar factories. It can be distinguished from *Bact. xylinum* by the multifarious forms of skin growth.

*Bact. vini acetati* has rounded, oval, and seldom moderately elongated cells, single or linked two and three together. Inflated cells also occur. The colonies on wort-gelatine are rounded, clear, have a moist glistening surface, and a whitish precipitate in the middle. The films are not very coherent, and the culture liquid soon turns cloudy. On wort with 3 per cent. alcohol the film has a greasy appearance and pale yellow colour. The optimum is at 28°-33°. As a nutritive fluid for these different bacteria, dilute vinegar with diluted beer or mash may be used, or diluted vinegar mash. In the vinegar factories, where the useful bacteria are attacked by

many foreign micro-organisms, attempts are made to suppress the dangerous kinds (mycoderma, mould, etc.), by the addition of about 2 per cent. of acetic acid.

Finally, an acetic acid bacterium may be mentioned, found by J. C. Holm on cocoa beans, which he has named *Bact. aceticum rosaceum*. It forms short, rounded, motionless rods 1.6  $\mu$  in length, single or in pairs. On wort or beer it forms a very weak, pale-coloured film, whilst the colonies on wort-gelatine and agar are distinctly pink in colour.

Acetic acid bacteria play an important part in the fermentation of beer, spirits, and wine. They do much harm, especially in wine, and if they once attain a strong development, the wine is irretrievably spoilt.

In low-fermentation breweries they usually do less mischief, as their growth requires a high temperature and an abundant supply of air. Thus, they are readily suppressed in a well-arranged lager beer cellar. Hansen's experiments have shown that *Bact. aceti* and *Bact. Pasteurianum* are able to exist during the whole time of storage, whether the infection takes place at the beginning or end of the principal fermentation. In his experiments the contamination, however, did not manifest itself during the whole course of the fermentation either by the taste or by the smell of the beer. When the beer was bottled, and exposed to a higher temperature, the bacteria developed further; yet, when the bottles were well corked, the beer did not turn sour. Just the same result was arrived at when the finished beer was infected. If, on the contrary, the bottles were badly corked, the growth turned the beer sour.

In high-fermentation breweries, on the other hand, where fermentation is carried on at higher temperatures, these bacteria are capable of doing much mischief, even before the beer leaves the brewery.

It is of practical interest to note that the species described by Hansen exert no influence on the colour or brightness of the beer, whilst most other bacteria cause turbidity.

In distilleries, and more especially in air-yeast factories, acetic bacteria may occur in large quantities, as shown by numerous experiments made by the author. They are most

frequently accompanied by mycoderma species. A careful control of the manufacturing process in this respect should never be omitted.

While investigating the influence of acids, especially acetic acid, on wine yeasts, Lafar found that each of the different acids (malic, tartaric, lactic, acetic, etc.) exerts a peculiar influence on the yeast, and not only on the proportionate amounts of alcohol and carbon dioxide produced, but also of glycerine; the acetic acid samples contained the smallest amount of glycerine and showed the weakest growth of yeast. Contrary to the previously accepted view that even small amounts of acetic acid prevent alcoholic fermentation, Lafar found that the presence of 0.27 per cent. had practically no influence on the rate of fermentation, the multiplying of the cells, or the yield of alcohol and glycerine. In must, before neutralisation, the yeast cells were not impaired by an addition of 0.74 per cent.; and in neutralised must, after adding as much as 1 per cent. of acetic acid, 4.77 per cent. by volume of alcohol was formed—*i.e.*, 60 per cent. of the maximum yield. Yeasts differ considerably, however, in their sensitiveness to the action of acetic acid. Thus, a comparison of fifteen different wine yeasts showed that all were able to carry on fermentation in the presence of 0.8 per cent. of acetic acid in a must that had previously been neutralised, whereas with 1 per cent. of acid only three were active. With regard to the propagation of cells, yeasts behave differently with the same amount of acetic acid. Lafar also examined the influence of these acids on the chemical activity of wine yeasts—*i.e.*, on the proportion between the amount of alcohol produced and the number of yeast cells formed. He found that in presence of 0.88 per cent. of acid the amount of work done by one cell was greater in the case of ten varieties, but smaller in two varieties, than in the presence of 0.78 per cent. Those yeasts, which are active in presence of 1 per cent. of acid, gave a smaller yield than in presence of 0.88 per cent.

According to W. Seifert, the nitric acid present in wines which have been diluted with water containing nitrates, is completely decomposed by the action of certain acetic acid bacteria.

## 2. Lactic Acid Bacteria.

If the micro-organisms of milk are subjected to spontaneous development at a temperature of  $30^{\circ}$ - $35^{\circ}$  C., the lactic acid organisms soon begin to ferment the lactose present (about 4 per cent.), and the acid produced protects the milk from putrefaction. After a certain quantity of acid has been formed it checks the activity of these bacteria, and the milk mould (*Oidium lactis*) develops. This oxidises a portion of the lactic acid, and thus enables the bacteria to restart their action. The same effect is produced if the acid is neutralised, for instance with calcium carbonate, and thus the complete fermentation of the milk sugar may be carried out. Simultaneously with the formation of lactic acid, casein, which forms the most important part of the albuminoid constituents of milk, separates out. Before souring, the casein occurs as a calcium salt (100 casein to 1.55 CaO), and is present in the colloidal form. When the milk is soured, the lime combines with lactic acid, liberating the casein, which is precipitated in a fine flocculent condition, causing the curdling of milk. In addition to this a greyish-yellow serum gradually separates out containing calcium lactate, lactose, albumen, etc.

Other varieties of lactic acid may be developed by exposing a malt or other mash to a given temperature. If the mash is maintained at  $40^{\circ}$  C. a pediococcus form develops vigorously, if at about  $50^{\circ}$  C. a short rod form. If a fraction of the liquor is transferred to another mash at the same temperature, the respective forms each receive an impetus, and after a few inoculations only the two forms can be discovered in the respective mashes by an ordinary microscopical examination. It will be noticed that by the process described there can be no guarantee that a pure culture has been obtained, for in each case other bacteria survive, even if in an extremely weak condition, and, on the other hand, there is a possibility that more than one species, or variety, of lactic acid bacterium may develop at each temperature. Similarly in beer-wort and other liquids spontaneous lactic acid fermentation may occur. This is also the case in the souring of "sauerkraut," the preparation of leaven, ensilage, etc., and the bacteria which develop in

these several fermentations doubtless represent many different species.

The lactic acid developed in the fermentation of milk, an acid first definitely characterised by Scheele in 1780, corresponds approximately with the quantity of lactose that has disappeared. Only minute quantities of by-products are formed, as was proved by the detailed researches of Kayser. If the fermentation is continued for some time, many species will decompose part of the lactic acid originally formed. Kayser found that a pure cultivated species from cream grown in lactose-peptone-wort had lost 0.26 grammes of lactic acid per litre in eleven days. If volatile fatty acids are formed, they will tend to increase in quantity under these conditions at the expense of the lactic acid. According to O. Jensen, lactic acid may itself be converted into volatile fatty acids.

Lactic acid formed by the spontaneous fermentation of milk is usually optically inactive—i.e., it does not turn the plane of polarised light either to the right or to the left. If, however, the active bacteria are isolated in pure cultures, and inoculated into sterile milk, species are developed which produce a lactic acid turning the plane of polarised light to the right (dextro-rotatory bacteria), and others producing a laevo-rotatory acid. The dextro-rotatory species occur more frequently. Thus the species of bacteria determines whether one or the other sort of acid shall be produced. It appears, however, that there are also species of lactic acid organisms in which the optical activity of the product of fermentation depends upon the composition of the nutritive fluid, as shown especially by Kayser. The species reacts differently with different sugars. Thus the common *Bact. lactis acidi* (Leichmann) ferments dextrose, lactose, maltose, mannite, and raffinose. Hueppe's *Bac. acidi lactici* ferments saccharose, dextrose, lactose, and mannite. A few species thrive best when they have access to atmospheric oxygen, whilst others carry on the fermentation equally well, or even much better, in the absence of air. They also exhibit differences in the rapidity with which acidification takes place at different temperatures. Thus, the *Bact. lactis acidi* has its optimum for the formation of acid at 32°-38° C., Hueppe's *Bac. acidi lactici* at 35°-42° C.,

others at 20°-22° C. and 40°-48° C. In general the largest amount of acid is generated at temperatures somewhat below the given optimum.

In 1903, Herzog proved that *Bac. acidi lactici* contains an enzyme which can be isolated from the living cell, and is capable of producing a lactic fermentation. He treated a pure culture mixed with kieselguhr with methyl alcohol, and afterwards with ether. The mass was then dried, and the resulting white powder, which contained no living cells, could convert minute quantities of lactose into lactic acid.

Buchner and Meisenheimer subsequently proved that if a culture of one of the species growing in a distillery mash, *Bac. acidifacans longissimus* (*Bac. Delbrücki*, Leichmann) is treated with acetone, whereby it is killed, and the mass is then dried, a powder is obtained which can bring about a lactic acid fermentation in a sugar solution.

It may, therefore, be assumed that all bacteria of this group contain enzymes that can bring about fermentation independently of the living cell.

To cultivate the lactic acid bacteria of milk, a preparation of peptonised milk made by O. Jensen may be used:—To a litre of sterilised milk, 10 c.c. of pure concentrated hydrochloric acid and 2 grammes of pepsin (*P. granulatum*) are added. The mixture is kept in an incubator, and frequently shaken. When the casein has dissolved, the acid is neutralised, and the liquid sterilised at 115°-120° C. Gelatine or agar may be added before neutralisation.

In addition to the proper lactic acid organisms, there are a large number of bacteria, and, amongst them, some pathogenic forms which develop this acid.

We are indebted to Pasteur for the first important work on the subject of lactic acid bacteria. In 1858 he described the species which appears when milk spontaneously ferments. In his *Études sur la bière* he depicts certain bacteria growing in wort or beer in which lactic fermentation has begun (Fig. 22); he describes them as short rods slightly narrowed in the middle, and commonly occurring singly, rarely united in chains. In 1877 Lister prepared a pure culture of a lactic acid bacterium from sour milk, which he called *Bacterium lactis*.

In 1884, Hueppe found a bacterium in a spontaneous lactic acid fermentation which converts lactose and other sugars into lactic acid with the simultaneous formation of carbon dioxide (*Bacillus acidi lactici*). It consists of short, plump, motionless cells, the length of which exceeds their breadth by at least one-half; they are united chiefly in pairs, and seldom in groups of four. In gelatine plates they form whitish colonies; those below the surface are stellate, uniformly dark, and sharply outlined; on the surface they appear as flat white glistening nodules, resembling porcelain surrounded by clear outer zones. Atmospheric oxygen is necessary for fermentation with this species.

In recent times a large number of species of lactic acid bacteria have been found in milk. Marpmann, in 1886, described five species embracing both coccus and longer and shorter rod forms, and showed that the whole series was capable of producing a slight formation of alcohol.



Fig. 22.—Lactic acid bacteria (after Pasteur).—In order to give an idea of the size of the bacteria, some yeast cells are figured among them.

Hueppe and Grottenfels have since described new species, of which Grottenfels's *Streptococcus acidi lactici* appears to be identical with the *Bact. lactis acidi* described by Leichmann.

Adametz and Freudenberg have isolated species from Emmenthaler cheese (*Bac. casei*) which are for the most part facultative anaerobes.

Leichmann has thrown new light on the conditions present during the spontaneous souring of milk. He found that a single species or type strongly preponderated, and named it *Bact. lactis acidi* (*Streptococcus lacticus*). He described it as consisting of short motionless rods about one and a half times as long as they are broad, sometimes present in pairs, sometimes in chains (the latter particularly when cultivated in sugar-broth). On gelatine plates, the immersed colonies consist of round discs, white or pale yellowish-brown, at first transparent,

afterwards opaque. The surface colonies develop with extraordinary difficulty; they are transparent, and have a somewhat irregular edge. This species excites fermentation even in complete absence of air; in presence of a full supply of air the fermentation is restricted. Both Leichmann and Weigmann consider this species to be the regular means of spontaneously curdling milk, and explain the process of souring as follows:—Hueppe's *Bac. acidi lactici*, and other species of the aërogenic group, remain in the upper layers of the milk, to satisfy their great demand for air, and acidify these; whilst the facultative anaërobe, *Bact. lactis acidi*, develops in the lower layers, and acidifies them. This species is easily grown in lactose- or grape-sugar-broth, and then forms long chains. As already stated, it ferments these sugars as well as lactose, mannite, and raffinose. It forms dextro-lactic acid, and produces no evolution of gas in sterilised milk.

A number of so-called species described by different authors appear to be varieties of this species. In spontaneously soured milk, Leichmann discovered another species of frequent occurrence, which has great similarities with the above, but may be distinguished by its production of laevo-lactic acid, and by the evolution of gas (*Micrococcus acidi levolactici*). He also isolated a species which thrives best at 44°-52° C., develops laevo-lactic acid, and forms thin rods of varied length. On agar it forms root-like, branching colonies. The great series of interesting varieties cultivated in a pure state by Weigmann deserves special attention. Their appearance at first resembles that of Leichmann's *Bact. lactis acidi*. Biologically, however, they show important differences, and several have found widespread industrial application. Other species have been described by Marpmann, Conn, Kozai, Beijerinck, etc. In addition to these typical lactic acid bacteria, other species occur in the souring of milk which possess a curdling and a peptonising enzyme.

The ripening of cheese, which consists in a conversion of casein (paracasein) into simpler albuminoids, and the breaking down of the latter, is brought about chiefly through the action of certain micro-organisms. As is well known, the curd may be separated from the milk by the addition of

rennet,\* whereby it remains sweet, or less frequently by the application of lactic acid (sour milk). The gradual decomposition of the curd is due to a slight extent to the action of pepsin contained in rennet, as well as to an enzyme (*Galactase*. Babcock and Russell), although, according to O. Jensen, this is precipitated from milk by bacteria at a very early stage. The action is, however, mainly due to the rich flora of micro-organisms embedded in the cheese. These are derived partly from the rennet, and partly, and, indeed chiefly, from the milk itself, and consist of lactic acid bacteria, peptonising bacteria, butyric acid bacteria, and moulds.

The basis of bacteriological work on cheese was laid by Cohn, Duclaux, and Benecke, and their researches have been extended by Adametz, Freudenreich, Weigmann, O. Jensen, and Harding. The main lines of this development must be regarded, according to these workers, as a modification of the albuminoids of the curd brought about during the first short period by peptonising bacteria—bacteria that had been active in the milk. Amongst these must be especially mentioned *Micrococcus casei liquefaciens*, which occur in great numbers. It multiplies at lower temperatures than the true lactic acid bacteria, and, therefore, is found in large numbers in the cooled milk, and is thus transferred to the cheese. It coagulates

\* Rennet is an enzyme which decomposes casein into paracasein and whey albumen. It is secreted in special glands of the stomach of various animals (e.g., the ruminants): calves' stomachs are used for the preparation of rennet. In the vegetable kingdom this enzyme is widely distributed. It is found, for example, in *Pinguicula*, *Ficus carica*, *Galium verum*, in the calyx of the artichoke (*Cynara scolymus*), and in many bacteria. In 1892, Conn isolated an enzyme resembling rennet from bacteria which were isolated from cream, and completely liquefied gelatine. They produced the enzyme most rapidly and freely at about 20° C. He isolated it from the filtrate of a ten-day-old milk culture. The filtrate was acidified with 0·1 per cent. of sulphuric acid, and then mixed with an excess of salt. A white foam separated out, which contained the comparatively pure enzyme. The dry foam formed a white powder. This enzyme also occurs in the ubiquitous putrefactive bacterium, *Bacillus vulgaris* (*Prot. vulg.*). It further occurs in *Bact. prodigiosum* (the bacterium of the Bleeding Host), and also in *Bac. coli communis*, which is always found in the intestines of men and animals, as well as in many of the "potato bacilli." Finally, it has been detected in torula species (*Lactomyces*), in different species of moulds (e.g., *Aspergillus*, *Monilia*), and in certain yeast species.

milk in 24 hours at 35° C., ferments lactose, and produces volatile acids, especially acetic acid. Its growth is, however, soon arrested by the true lactic acid bacteria, and, in particular, by the development of *Bact. lactis acidi* (*Streptococcus lacticus*), which causes a vigorous formation of acid. According to Weigmann, the lactic acid is then gradually displaced by acid-consuming fungi or by bacteria producing alkali, and the peptonising or casease bacteria come into activity; in particular, the species producing cheesy aroma.

The special characters of different kinds of cheese are due to special micro-organisms. The particular part played by the lactic acid bacteria in the process is to prevent the peptonising bacteria from getting too great a hold, and thus producing too quick a decomposition of the curd. The conversion process is thus regulated by means of the lactic acid organisms. According to O. Jensen, they further stimulate the action of pepsin derived from rennet. Lastly, their importance in the ripening of cheese depends to a great extent upon the fact that certain species effect a further transformation of the products of decomposition, especially of the albumoses and peptones produced in milk, and in the early stages of the ripening of cheese by peptonising bacteria. In particular, the production of volatile acids, such as propionic acid and acetic acid, detected by O. Jensen, is to be attributed to this cause. Quite recently Jensen has discovered a special propionic acid ferment in Emmenthaler cheese, which he believes to be a variety of *Bact. lactis acidi*. According to Jensen the carbon dioxide produced by this species forms the "eyes" in Emmenthaler cheese.

Amongst the casein-digesting bacteria must be classed the aërobic *Tyrothrix* species, minutely described by Duclaux, thread-forming bacteria, which secrete an enzyme resembling *trypsin*, and belong to the group of hay bacilli. To this group belongs *Bacillus nobilis*, discovered by Adametz, and, lastly, *Paraplectrum foetidum*, detected by Weigmann in Limburger cheese, which occurs in milk as thick rods, and at the temperature of the incubator (30°-40° C.) assumes mallet shapes, and quickly forms spores which are twice as long as they are wide.

Anaërobic bacteria also play a part in cheese fermentation, and, amongst them, butyric acid bacteria.

Amongst moulds which are of special importance in determining the character of different cheeses must be mentioned the *Mucor*, *Penicillium* and *Dematiuim* species observed by O. Johan-Olsen in Norwegian "Gammelost" (old cheese); a white *Penicillium* (*P. candidum*, Rodger) in Camembert cheese; and a similar *Penicillium album* (Epstein) in Brie cheese. Weigmann, Conn, and others, on the contrary, attribute the special character of both these cheeses to the action of a particular *Oidium*. O. Jensen assumes that the peculiar flavour of Roquefort cheese is due to the symbiosis of *Penicillium glaucum* and *Oidium lactis*. All these moulds act partly by decomposing the acid contained in the cheese, and partly by neutralising the acid with ammonia formed by the breaking down of casein, and thus they prepare the ground for the peptonising bacteria, whilst also decomposing the milk fat, and liberating its volatile fatty acids.

The abnormal characters of milk and milk products must in an equal degree be attributed to micro-organisms.\* Thus, for instance, the bacteria introduced into milk from a diseased udder, and the consequent changes in the character of the milk, are accompanied, not only by a very great increase in the bacterial contents, but also by the presence of characteristic pus cells in the milk.

"Soapy" milk, having a decided soapy taste and producing a strong lather, owes these properties to the presence of *Bac. lactis saponacei*, a short rod which forms slimy colonies on ordinary nutritive gelatine, turning to a rusty yellow on the surface. Other species may also produce this fault.

Bitter milk may be the result of using certain food-stuffs, but may also be produced by certain bacteria, as was shown by Pasteur, Duclaux, Löffler, Weigmann, and others. In practice, micrococci which liquefy gelatine and certain varieties of aërobic, lactic acid bacteria appear to have this effect.

\* It is generally accepted that milk may act as a carrier of many dangerous disease germs; typhoid epidemics in particular appear to spread in this way. Tubercular bacilli, capable of development, have frequently been found in raw milk.

Certain *Torula* yeasts may grow in milk and make it bitter (Calloghan and Harrison). "Ropy" milk has a marked slimy character, and can be drawn out into threads. This is due either to the enormously swollen membrane of certain bacteria, or to the formation of slimy albuminoid bodies. The active micro-organisms are either varieties of lactic acid bacteria, or more particularly the bacteria that digest albumen. A widely distributed species is *Bac. lactis viscosus*, described by Adametz, which gradually converts the viscid milk into a substance resembling honey. Another widely distributed and active species is *Micrococcus Freudenreichii*, described by Guillebeau, which liquefies gelatine. The lactic acid bacterium, *Streptococcus hollandicus*, Hueppe, described by Weigmann, Goethart, Boekhout, and others is of particular interest; it occurs in Dutch "lange Wei" (aropy cream used in the manufacture of Edam cheese). This organism is a facultative anaërobe, and has its optimum at 21°-22° C. Like many other varieties it easily loses its property of forming slime. A similar variety was found by G. Troili-Petersson in the Swedish "tätmjölk" (thick milk), and described under the name of *Bact. lactis longi*. It is believed that this species constructs slime from lactose. It has its optimum below 20° C. Probably such bacteria are present on certain plants that are placed in the milk, such as *Pinguicula* and *Drosera*.

By the action of micro-organisms milk may assume a blue, red, or yellow colour. The blue coloration of milk depends upon the growth of certain species of bacteria, the presence of which was proved by Fuchs as early as 1841. Hueppe was the first to prepare a pure culture of one of these species, and he described it under the name *Bacillus cyanogenus* (*Bact. syncyanum*), which occurs as a short motile rod. In the case of this, the most widely distributed species, the colour appears first on the surface of the raw milk, and afterwards penetrates to the lower layers. The production of colouring matter originates from the albuminoids, and may occur in the absence of sugar. A number of the water bacteria also have the power of imparting a blue colour to milk. The reddish colour which milk occasionally assumes is also due in certain cases to bacteria. Only a few examples are quoted in

the technical literature. Hueppe found a *Bact. lactis erythrogenes* in red milk, which is described by Grotenfelt as a short rod coagulating milk, and producing a red colour on gelatine plates. Menge found a *Sarcina rosea* in red milk, which also forms red colonies on gelatine, and a few other species with similar properties have been detected. In yellow milk a *Bact. synxanthum* has been observed and described by Schröter.

The taints observed in butter must also be chiefly attributed to micro-organisms, and in the technical literature it is strongly emphasised that a great development of such harmful species is frequently caused by a want of cleanliness, or by a wrong souring of the milk. The rancidity of butter, which is due to the presence of butyric acid and ethyl butyrate, is caused by the action of light and air. According to O. Jensen this phenomenon is due to the presence of aërobic fungi, which cause the decomposition of fat, in particular *Cladosporium butyri* and *Oidium lactis*. This may further be due to the presence of two bacteria universally found in water, *Bac. fluorescens liquefaciens* and, occasionally, *Bact. prodigiosum*. The action of light may also produce the tallowy taste, but Storch has isolated a rod-shaped, lactic acid bacterium which can produce the same effect. A turnip flavour and a rotten-sweet flavour in butter, studied by C. O. Jensen, proved to be derived from a special species, *Bacillus foetidus lactis*. Weigmann has subsequently observed similar bacteria. A whole series of other irregularities in the character of butter are also accompanied by the growth of different micro-organisms, and the assumption is reasonable that they must be the originators of these taints.

One of the most pronounced faults with cheese is "blowing," which is due to the presence of great masses of fermentation bacteria causing an excessive development of gas; in particular, a species, *Bac. Schafferi*, belonging to the *Bac. coli* group, plays an important part in this respect. The same fault may be produced in cheese by the species originating from diseased udders. Yeasts may produce a strong evolution of gas and certain varieties of *coli* and *aërogenes* species may also bring about vigorous fermentation with production of gas. A

suitable degree of souring with lactic acid bacteria appears to be a certain means of preventing these mishaps.

The blue flecks which appear in certain kinds of cheese may be produced, according to Beijerinck, in some cases by *Bac. cyanofuscus*, which is derived from water. Black flecks may be caused by growths of moulds, such as *Cladosporium* and *Fumago*. Rusty specks, according to Connell, Harding, and other American observers, are caused by a definite species, *Bac. rudensis*. In the same way a reddish colour is produced by red moulds, micrococci, etc.

Lastly, a fault must be mentioned which may occur in all kinds of cheese, the bitter taste, which is caused by certain bacteria, as, for instance, by *Micrococcus casei amari*, described by Freudenberg, and also by a species occurring in bitter milk, and even by certain moulds and *Torulae*.

Since 1890 methodically selected species of bacteria have been applied in dairies, to bring about a regular and certain souring of the cream used in the manufacture of butter, and to avoid any taint in butter. The progress made in this field is associated with the researches of Storch, Weigmann, Quist, in the author's laboratory, and others. The pure culture selected is added to skim milk, previously heated to about 90° C., and the culture is allowed to develop at about 15° C. After standing 24 hours, this "starter" is fit for use. In order to render the cream which is to receive the culture as free from germs as circumstances permit, it is pasteurised at about 85° C., and then quickly cooled. In the course of ten hours or so, the starter is allowed to develop in cream at about 16° C. It is then cooled below 10° C., and churning is begun.

Among the forms isolated by Storch of Copenhagen (1890) from butter, sour cream, and butter-milk, the coccus form of the group *Streptococcus lacticus* seems to be most frequent and best suited to sour the cream. It occurs in a large number of varieties, which, according to their main characteristics, may be classed in two groups—one including those which give a specially pure and mildly sour taste and a fine aroma, and another embracing those which yield a product possessing great keeping powers. Morphologically the growths are dis-

tinguished from each other by the fact that some are connected in chains, others are not (Fig. 23); the latter are of the most frequent occurrence, and are most widely distributed. These forms bear a certain resemblance to Pasteur's "ferment lactique." The species represented in Fig. 23, B, was isolated by Storch from a sample of butter having a pure and full aroma. It forms small globular colonies in gelatine of a pure white colour and smooth surface. In milk and whey it occurs in oval or globular forms. These lactic acid bacteria display fermentative activity, even at 20° C. At 28° C. milk is turned sour within eight to nine hours.

Many species have been isolated by Weigmann and intro-

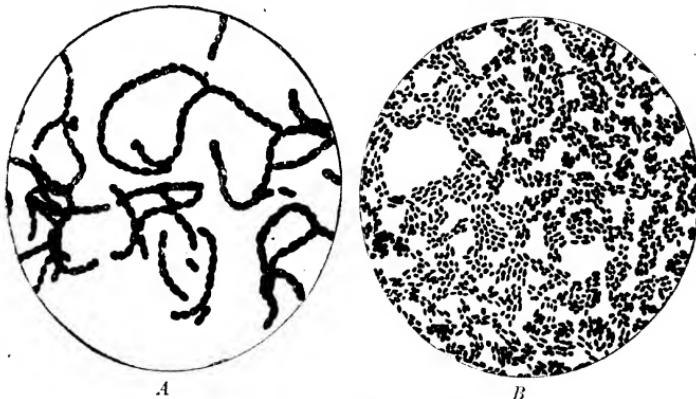


Fig. 23.—Lactic acid bacteria (after Storch).

duced into practice. A species which has been very successfully applied at several places was prepared by Quist, and afterwards by J. C. Holm in the author's laboratory. It occurs both as micrococcus and in other forms, according to the different nutrient media in which it is cultivated. On gelatine it forms small, circular, slowly-growing colonies of a whitish-yellow colour. In stab-cultures spherical colonies arise throughout the puncture-channel, and in streak-cultures this organism forms a continuous streak with wavy borders. It was prepared from a sample of butter of remarkable aroma and durability.

Pure cultures of lactic acid bacteria have also been applied

in cheese factories to regulate the ripening of cheese. They are always added to raw milk, as it is of importance that other species of bacteria from the milk which play a part in the process should not be suppressed. Attempts have also been made to apply cultures of other bacteria and of moulds in the preparation of cheeses of pronounced character—e.g., Roquefort, Camembert, etc.

As the mash in **distilleries** is not allowed to exceed a temperature of  $70^{\circ}$  C., in order that the diastase may be preserved, many of the germs adhering to the raw materials are not killed, but are capable of developing during fermentation, and thus they may not only utilise the nutritive substances, but also disturb the desired alcoholic fermentation: in the latter respect, butyric acid bacteria are specially dreaded. With the view of preventing too strong a development of germs injurious to the yeast, various acids have been added direct to the mash, or else a lactic acid fermentation has been previously carried out in a fraction of the mash. Thus a tenth part may be kept at a temperature of  $50^{\circ}$ - $55^{\circ}$  C., till it shows about  $2\frac{1}{2}^{\circ}$  of acidity,\* corresponding to about 1 per cent. of lactic acid. At this temperature the desired species of lactic acid bacteria develop, whereas it is too high for the majority of bacteria. An excellent means for maintaining the mash at this temperature is the acid chamber introduced by Kruis into distilleries, a small and well-isolated space in which the air maintains a constant temperature, and in which the mash to be soured is introduced and allowed to stand quietly as soon as it has cooled down to the same temperature. The mash is then heated up to  $70$ - $75^{\circ}$  C., whereby part of these bacteria are killed. After subsequently cooling down to about  $20^{\circ}$  C., the yeast is added. The yeast is not affected by this quantity of lactic acid. After it has developed sufficiently, the mixture is employed for pitching the principal mash. To devise a rational process for pitching the lactic acid mash with bacteria, a part of the mash must be placed on one side before yeast is added, and used to start the

\* *i.e.*,  $2\frac{1}{2}$  c.c. of normal caustic soda solution are required to neutralise 20 c.c. of mash.

souring of the succeeding acid mash. About one-tenth of the fermented acid mash is used for pitching the following mash.

The acid thus introduced into the principal mash, together with the surviving lactic acid bacteria, act as disinfectants, besides exerting an influence on the yeast cells, both directly and by reacting on the nutritive substances.

The lactic acid bacteria occurring in the mash can be distinguished in many ways from those occurring in milk. Zopf was the first to prepare and investigate a culture of a species belonging to this class, from a mash obtained from dry malt and water at 50° C., according to Delbrück's process (1881), following up an observation of Delbrück's that at this temperature a vigorous lactic souring took place. A growth of threads, rods, and cocci was developed.

*Pediococcus acidi lactici*, examined by Lindner, gives a strong acid reaction when cultivated in a neutral malt-extract solution at 41° C. Both in a solution of this kind and in a hay decoction, which has not been sterilised, this bacterium develops so vigorously that, according to Lindner, all other organisms are suppressed at this temperature. It has been proved chemically that the acid, which is abundantly produced, consists for the most part of lactic acid. When a malt mash or malt-rye mash is maintained at 41° C., the *Pediococcus* develops vigorously, and the rod-shaped lactic acid bacteria are suppressed. According to Henneberg the optimum for the formation of acid is 38° C. The optimum for growth on beer and wort agar lies between 36° and 40° C. In a neutral malt-extract solution the *Pediococcus* is killed after five minutes' exposure to 62° C. In gelatine it does not thrive well; it is only in stab-cultures in neutral malt-extract gelatine that very vigorous white colonies are formed below the surface. This species appears, on the whole, to thrive better when air is excluded.

In 1893, Kruis and Rayman isolated a vigorous lactic acid bacterium from yeast mash consisting of long and short rods, which produced 0.9 per cent. of lactic acid at 40° C. in a clear malt wort. It is of special interest to note that Kruis and Rayman in studying this species proved, for the first time, that lactic acid bacteria are capable of forming volatile fatty acids.

Lafar isolated from the sour yeast mash a species which he named *Bac. acidificans longissimus*, and since 1894 it has been applied in practice for souring the yeast "goods." It ferments saccharose, galactose, dextrose, lævulose, and maltose, but not lactose, and it occurs both in short rods and in very long threads. A short time afterwards, Leichmann described a bacterium occurring under similar conditions, *Bac. Delbrücki*, which is believed to be identical with Lafar's species. It shows great resemblance to Leichmann's *Bact. lactis acidi*, and both species produce lævo-lactic acid. It cannot, however, like the latter, ferment lactose. In a lactose broth it produces no acid, and grows with difficulty, whereas in grape-sugar broth or maltose broth, as well as in sweet wort, it grows vigorously. According to Henneberg this species has its optimum for acid production at 46°-47° C. In the mash it forms up to 1.79 per cent. of lactic acid. The amount of acid is reduced with free access of air. Its optimum for growth lies between 40°-48° C. In the mash, it occurs with both short and long cells, single or grouped two and three together. On solid substrata it forms small, flat, clear colonies.

Henneberg has isolated a number of other species of lactic acid organisms from mash and pressed yeast, which he has described as "wild," some of which may produce direct damage in the industry, if care is not taken to secure a vigorous yeast fermentation, for they not only carry on the production of acid throughout the fermentation, but form at the same time volatile acids, especially acetic acid, which damages the yeast and reduces the output of alcohol. Other members of this group appear to be harmless.

All the species examined grow and produce acid in presence of yeast at 27.5°-30° C. Amongst the dangerous kinds may be named *Bac. Hayducki*, which occurs in mash in small short cells, mostly single, and forms round white colonies on gelatine (its optimum for acidification is first at 45°-46° C.; later at 33°-35° C.), and *Bac. Buchneri*, with similar cells in the mash and white or yellowish colonies on gelatine (optimum for acidification first 39°-40°, and afterwards 23°-30° C.).

There appears to be no doubt that the lactic organisms occurring in the mash have a tendency to variation, and that

not a few of the numerous species described must be regarded as sub-species of a certain parent form.

Thus Beijerinck takes, as the parent form of Leichmann's species, *Bac. Delbrücki*, a very vigorous acid producer *Lactobacillus fermentum*, a young culture of which prepared at 37° C. for 36 hours in an acid mash, and then transferred to wort jelly (gelatine agar), forms very small transparent colonies consisting of motionless bacilli of different lengths together with micrococci. In wort it forms threads of different lengths. The smaller the amount of oxygen present the more extended are the cells. Its optimum for the production of acid is 41°-42° C., and at 50° C. it ceases to develop acid. According to Beijerinck this parent form may be converted into *Bac. Delbrücki* through a continued series of cultivations with free access or air at 48° C. (well above its optimum for acid production), and then transferring to wort jelly at 37° C.

In the distillery, Lafar, Leichmann and the author (in 1896), and more recently Kusserow and others, have introduced into practice pure cultures of predominant species occurring in normal sour mash (*Bac. acidificans longissimus*, *Bac. Delbrücki*), with the object of regulating the souring of the mash. By the proper application of such cultures in distilleries and yeast factories, and at the same time securing a uniform temperature of acidification throughout the whole mash, it is possible to absolutely prevent the development of foreign and harmful bacteria.

In the brewing industry the lactic acid fermentation takes place in malting, and in particular in mashing. Accurate investigations were made by Prior. Some lactic acid is also formed during fermentation. Lactic acid is formed in large quantity in Belgian beers prepared by "spontaneous fermentation," imparting a sharp taste. "Weissbier" owes its refreshing taste to a vigorous lactic acid fermentation. In modern low-fermentation breweries attempts are made to get rid, not only of lactic acid bacteria, but also of bacteria of all kinds from the fermentation.

*The Saccharobacillus Pastorius* described by van Laer, which occurs in the form of rods of different lengths, produces a characteristic disease ("tourne") in weakly hopped beer,

which manifests itself as follows :—The liquid gradually loses its brightness, and when it is agitated filaments of a silky lustre rise from the bottom, and the beer assumes a disagreeable odour and taste. According to Fellowes, this species is also found in English beer. It does not always react\* on the beer, probably on account of the larger amount of hops. In cultures, the bacillus develops either in the presence or absence of free oxygen. In nutrient liquids it ferments carbohydrates, and amongst them the saccharoses, without previously inverting them. Amongst its fermentation products, lactic acid, acetic acid, and alcohol predominate. The acids produced cause the precipitation of nitrogenous compounds in the liquid, and these, mixed with the bacilli, produce a cloudiness, consisting of lustrous filaments. The nutritive mixture best suited to this bacterium is an extract of malt mixed with agar and a small quantity of alcohol, or, still better, neutral or slightly alkaline sweet wort.

If this bacterium is exposed to a temperature of 55°-60° C. in beer, it is soon killed. Henneberg has closely investigated both this species and two other lactic acid bacteria occurring in beer which cause the same disease—*Saccharobacillus Past.*, var. *berolinensis* and *Bac. Lindneri*. These all occur as thin and comparatively long bacilli, either straight or curved, and usually cluster together. In hanging drops they form very long threads. From a physiological point of view they behave quite differently. *Saccharobacillus Past.* gives the most vigorous formation of acid in arabinose and trehalose, weaker in saccharose, maltose, dextrose, lævulose, and galactose. Its optimum for acid formation lies between 24° and 33° C. The variety *berolinensis* gives a weaker yield of acid, and none at all in raffinose and trehalose. Its optimum for acid formation is at 20°-24° C. According to Henneberg it is this species in particular which grows in Berlin "Weissbier," and imparts to it its peculiar character. Other varieties of lactic acid bacteria occur, however, in this kind of beer.

*Bac. Lindneri* produces acid in maltose, and to a small degree in dextrose. The optimum for acid formation is at 17°-18° C. This species or variety frequently occurs in ordinary lager beer, and influences its flavour and aroma.

without, however, producing any considerable amount of acid. It may occur in the form of long cells in lager beer.

Schönfeld observed a species in various high-fermentation beers, which looks like the bacterium of Berlin Weissbier, and gives to these beers a slight lactic acid flavour, and may make the beer membranous.

In the fermentation of wine lactic acid bacteria may also occur, and may produce great alterations in the constitution of the liquid. Amongst the better known phenomena are "vin tourné" and "poussé" (lactic acid, Zickendwerden), due to the presence of considerable quantities of lactic acid.

Pasteur, as early as 1866, referred these diseases to the activity of bacteria, and at a later date Müller-Thurgau proved that a short bacillus regularly occurs in such wine, and is capable of converting many of the constituents of the wine into lactic acid, or of producing a lactic acid fermentation in wine must. The disease declares itself in this way:—The wine becomes turbid, and is at first pale, afterwards dark, and deposits a sediment. At the same time it assumes an unpleasant smell and taste. The disease appears in those seasons when the must is poor in acid, so that the bacteria find more favourable conditions for growth. A bacterium may be mentioned here that was isolated by Gayon and Dubourg, a rod-shaped motionless organism which occurs especially in red wine, and forms mannite (*ferment mannitique*), whereby the wine assumes a characteristic bitter-sweet taste. By the fermentation of different sugars it forms lactic acid, acetic acid, etc., and only forms mannite by fermentation of levulose. It grows freely in dextrose and broth, and has its optimum at about 35° C. By heating the wine to 60° C. its development is restricted, and this is also the case if the fermentation is vigorously carried out below 30° C. Like the previous species its development is favoured by a wine with a low acid content. By the addition of tartaric acid its growth may be checked. *Bact. mannitopaeum*, discovered by Müller-Thurgau, belongs to this group. It forms the bacterial bubbles described in an earlier section (*zooglaea*). It occurs in the form of motionless rods, both long and short, and threads, which may produce snow-white flecks in fruit wine. In

must-gelatine the colonies are round or sausage-shaped, white, and non-liquefying. The species is facultative anaërobic, and has its optimum for growth at 25°-30° C. It decomposes lävulose and saccharose, and forms acetic acid, lactic acid, and mannite. According to Müller-Thurgau's observations, mannite fermentation occurs fairly frequently in fruit wines, especially those prepared from over-ripe fruit, lacking in acid. It is accompanied in fruit wines, as well as in grape wine, by the formation of larger quantities of lactic and acetic acid.

In this connection a species must be mentioned which plays an important part in the reduction of acid in wine. According to Wortmann, Alfred Koch, and Seifert, certain bacteria bring about this result, and Seifert has isolated a species in pure culture. It is named *Micrococcus malolacticus*, and destroys part of the malic acid, the most important acid of the wine, and forms lactic acid. As a consequence of the activity of this bacterium, the wine loses its acidity, and acquires a milder flavour, causing an improvement in its quality. Like other organisms that destroy acid, its activity is first displayed after the proper fermentation has ceased and the yeast is in a weak condition. The species has its optimum at 25°-34° C., and the limits for its growth are at 34°-37° C. It forms small milk-white transparent colonies on nutrient gelatine.

Other bacteria forming lactic acid and producing diseases in wine are described by Mazé and Pacottet, as well as by Laborde, who has also investigated the mannite ferment.

In **leaven**, lactic acid bacteria also occur which, without doubt, play a part in the fermentation of bread. Peters, for instance, found a species which occurs in motile rods, and forms a slimy skin on neutral yeast-water-sugar at 30° C. Henneberg found Leichmann's *Bact. lactis acidi* by development in mash at 48° C., and at 38° C. a special species which he named *Bac. panis fermentati*, which occurs in mash in short and long rods, and forms small white colonies on wort-agar. The optimum for the production of acid is at first about 37°-42° C., and afterwards 34°-38° C. Henneberg also found different species of lactic acid organisms in pressed yeast along

with the proper culture species, and amongst them *Bac. Hayducki* and *Bac. Buchneri*. *Bac. Listeri* should also be mentioned, which occurs in comparatively short rods and in chains. Its optimum for acid formation is at 34° C. *Bac. Wörtmanni* has its optimum at 33°-40° C., and later at 25°-29° C. Lastly, three forms occur, *Bac. Leichmanni* (I., II., and III.). When cultivated in mash they appear chiefly as short rods linked in chains, with an optimum for acid formation of 35°-36.5° C. at first, and afterwards at considerably lower temperatures. When pressed yeast turns soft, the various kinds of lactic acid bacteria increase largely in numbers.

The lactic acid fermentation plays a very important part in the means adopted in different countries for **preserving vegetable foods** for both man and beast. Vegetables are chopped up, in certain cases salt is added, and they are placed in vessels or in hollows protected from access of air. A fermentation sets in and lactic acid is produced as one of the products. This acid protects the material from the attack of other micro-organisms, and gives the peculiar character to the preserved vegetables. The temperature usually rises when fermentation begins, which allows of the partial development of special thermophilous bacteria. As a consequence of the development of other micro-organisms, the amount of acid is always reduced with prolonged fermentation. The active species are described by Wehmer, Aderhold, Weiss, Henneberg, and others. A rich flora of species occurs, however, in such ferments. In the same way such a souring is used for the preservation of different feeding stuffs. The heat evolved during the fermentation imparts a special character to the fermenting mass, which varies according to the extent to which the temperature rises.

### 3. Butyric Acid Bacteria.

When stale milk in which lactic acid bacteria have developed is neutralised by the addition of calcium carbonate, so that calcium lactate is formed, it will, as a rule, undergo a butyric fermentation. Pasteur showed in 1861 that this fermentation is brought about by particular micro-organisms

which are able to live without air ("*vibrions butyriques*"). This spontaneous butyric acid fermentation takes place most vigorously at 35°-40° C. Starch, glycerine, dextrin, cane-sugar, maltose, lactose, and dextrose are likewise decomposed by the butyric acid ferments, and such fermentations are of frequent occurrence, as the bacteria belonging to this group are very widely distributed in nature. In order to induce a butyric acid fermentation, Fitz recommends using a mixture of 2 litres of water, 100 grammes of potato-starch or dextrin, 1 gramme of ammonium chloride, the ordinary nutrient salts, and 50 grammes of chalk; this mixture is to be maintained at 40° C. Bourquelot recommends exposing slices of raw potatoes, standing in water for two or three days at a temperature of 25°-30° C. (a temperature of 39° C. is more favourable).

A convenient process for assisting the growth of butyric acid organisms is given by Beijerinck as follows:—5 per cent. of finely ground fibrin is added to a 5 per cent. solution of grape sugar. After vigorous boiling, it is inoculated with garden soil, and immediately placed in an incubator at 35° C. The fermentation will set in within a day or two. The liquor is then neutralised with soda solution. A growth is thus obtained of Beijerinck's *Granulobacter saccharo-butyricum*, the majority of other bacteria being destroyed by boiling, or else checked by the butyric acid fermentation. If, instead of grape-sugar, cane-sugar is used together with 3 per cent. of calcium carbonate, 0.05 per cent. of sodium phosphate, 0.05 per cent. of magnesium phosphate, and 0.05 per cent. of potassium chloride, the *Clostridium* form develops in the liquor. Botkin's process is also worthy of mention; it consists simply in heating milk in closed flasks in a current of steam for half an hour, and then maintaining it at 35° C.

The most important products of the butyric acid fermentation are butyric acid, carbon dioxide, and hydrogen..

In the saccharine mashes of breweries, distilleries, and pressed-yeast factories, species of butyric acid bacteria always occur, and if the mashes are maintained for a lengthened period at certain temperatures, they develop very rapidly, and exercise a retarding influence on the alcoholic ferments.

According to Pasteur's experiments, the butyric acid ferment can perform its functions without access to free atmospheric oxygen. The usual spontaneous butyric acid fermentations proceed most vigorously when oxygen is excluded. It has, however, been shown by recent experiments that there are many butyric acid bacteria which behave otherwise regarding free oxygen, for some are incapable of growth in presence of oxygen—anaërobic species—whilst others multiply and induce butyric acid fermentation when they have access to oxygen—aërobic species. In the course of years a very large number of butyric acid bacteria have been described. By the study of this mass of material, it has been shown that they are divisible into two groups—first, the **true butyric acid bacteria**, being those that produce butyric acid as the chief product of fermentation by decomposition of carbohydrates or calcium lactate; and, secondly, there remain many species which form butyric acid along with other products by the breaking down of albuminoids. This applies particularly to putrefactive bacteria, many of which only produce minute quantities of butyric acid. Thorough chemical investigations have been carried out by Fitz, and more recently by Perdrix, as well as by Schattenfroh and Grassberger, who investigated the action of a number of species upon starch, the sugars, glycerine, cellulose, and the albuminoids, and determined the products of fermentation.

One of the first species to be minutely described is Prazmowski's *Clostridium butyricum* (*Bac. butyricus*, Fig. 24). It occurs in the form of short and long threads and rods, which may be either straight or somewhat curved. The rods are in brisk movement, and under a strong magnifying power they are seen to be covered with a large number of cilia (Fig. 25). Before the formation of spores in the rods, the latter swell and form peculiar spindle and lemon-shaped, elliptical, or club-like forms, as shown in the diagram; at the same time they are coloured blue by iodine. The spores can withstand boiling for five minutes. On germination the spores burst their outer envelope, and the germ filament grows in the same direction as the longitudinal axis of the spore. *Clostridium butyricum* grows most vigorously at a temperature of about

40° C., and may then rapidly become predominant in sugar solutions if the lactic acid ferment has previously converted a portion of the sugar into lactic acid. This species is decidedly anaërobic.

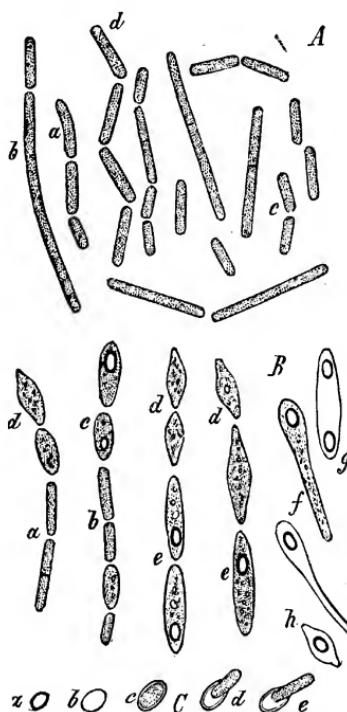


Fig. 24.—*Clostridium butyricum*, Prazm. (after Prazmowski).—A, Vegetative state; c, short rods; d, long rods; at a and b, rods and filaments curved like vibrios. B, Formation of "resting spores"; b, d, rods, previous to, c, e, during, f, g, h, after the formation of resting spores; e, elliptical; d and h, lemon-shaped; e, g, spindle-shaped; f, tadpole-form; at a rods still in their vegetative state. C, Germination of resting spores; the spore a expands into b; c, shows the differentiation of the membrane into exo- and endosporium. The contents surrounded by the endosporium issue from the polar fissure of the spore in the form of a short rod (d), which appears prolonged at e.

Fitz has described a species belonging to the aërobic organisms—a bacillus of a short cylindrical form, which is not coloured blue by iodine, is motile in a moderate degree, and forms no spores. It ferments all carbohydrates with the exception of starch and cellulose.

According to Fitz the spores of butyric acid bacteria can withstand the temperature of boiling water for a length of time, naturally dependent, as in all cases, on their condition and on the nature of the substratum ; Fitz gives three to twenty minutes as limits. They can, however, be killed at a lower temperature if maintained long enough ; thus they are killed by being heated for six hours at 90° C. in a solution of grape-sugar ; but in glycerine, at the same temperature, for a period varying from six to eleven hours.

Hueppe has likewise described a species (*Bac. butyricus*) found in milk, and occurring in the same forms as the species discovered by Prazmowski, but it proved much less sensitive to oxygen, and must, therefore, be classed as an aërobie. This species does not, however, form butyric acid from carbohydrates, but from albuminoids.

Another aërobie species, *Bac. booco-pricus*, was detected by Emmerling in cow dung. It forms short rods, and is characterised by not liquefying gelatine, and by converting glycerine into butyric acid.

Gruber found three well-defined species associated under the name of *Clostridium butyricum*, two of which are exclusively anaërobic. One of the latter species consists of straight or slightly curved rods, which become spindle- or barrel-shaped during the formation of spores. In nutrient gelatine it forms colonies which, when seen in reflected light, appear brownish-black or black. The second species consists of strongly-curved vegetative rods, at the end of which spores appear ; it forms yellowish or yellowish-brown colonies. The third species is also capable of growth and of causing fermentation in the absence of oxygen ; its development is, however, decidedly assisted by the presence of oxygen, and it is only then able to produce spores. The vegetative rods are cylindrical ; with the formation of spores the rods become spindle-shaped, and in the centre of the spindle the large spore is formed. The colonies in nutrient gelatine are yellowish.



Fig. 25.—*Clostridium butyricum* (after A. Fischer), with endospore and stained cilia.

All three species form butyric acid and butyl alcohol from carbohydrates.

In the water supply of Paris, Perdrix found an anaërobic bacterium (*Bacille amylozyme*), which occurs in the form of motile threads, four to six times as long as they are broad. This converts saccharose into acetic and butyric acids, with evolution of hydrogen and carbon dioxide, and it also produces amyl and ethyl alcohols. The optimum for its growth is 35° C. On slices of potato it forms whitish round colonies, which gradually liquefy the substratum. This species is very sensitive to acids. The spores can withstand ten minutes' heating to 80° C.

*Bac. orthobutylicus* was isolated by Grimbart from seeds of the leguminosæ. It was separated by heating for one minute at 100° C. Its spores survive this treatment. The species is anaërobic, and forms motile rods two to four times as long as they are broad, with rounded ends. In these rods two and three spores may occur. Its fermentation products are chiefly butyric acid, butyl alcohol, and acetic acid, together with carbon dioxide and hydrogen. It ferments saccharose, maltose, lactose, and glycerine. It hydrolyses starch, and converts dextrose into maltose. Grimbart proved by detailed experiment that the length of fermentation, the concentration, the reaction of the liquid, and the conditions of growth all influence the amounts of the fermentation products. Thus with an acid reaction the amount of alcohol increased and the formation of acid simultaneously decreased. On the other hand, the quantity of alcohol diminished and that of acid increased when the liquid was neutralised with calcium carbonate.

Amongst other workers in this field may be mentioned Botkin and Flügge, who isolated a species from milk by heating for one and a-half hours in boiling water or in a current of steam, the spores surviving this treatment; v. Klecki, who cultivated *Bac. saccharobutyricus* from cheese; v. Hibler, who examined pathogenic varieties, and proved that one and the same species may occur in different shapes not only as individuals, but also in colonies, according to the nature of the substratum. By cultivation in gelatine without sugar the different forms of colonies may be most clearly distinguished.

*Clostridium Pasteurianum*, discovered by Winogradsky, is of particular interest. He isolated it from garden soil by heating for ten minutes at 75° C., and then cultivating in a stream of nitrogen in a substratum free from nitrogen. The species can, therefore, absorb free nitrogen from the air and assimilate it. It can, however, utilise nitrogen in combination. It forms butyric acid, acetic acid, minute quantities of alcohol, carbon dioxide, and hydrogen, and occurs as short, thick motile rods, which at a later stage expand into spindle shapes, and during the production of spores gives a violet-brown colour with iodine. The free spores are surrounded by an irregular mass of jelly.

Beijerinck has drawn special attention to the possibilities of butyric acid bacteria which display bodies resembling granulose in the swollen cells and may be coloured with iodine, and has formulated a group which he calls *Granulobacter*. It consists of a series of species, some of which are identical with those previously described. The true originator of the butyric acid fermentation, the preparation of which has been described, he calls *Granulobacter saccharobyticum*; it forms varying quantities of butyl alcohol, carbon dioxide, and hydrogen from saccharose, better from glucose, and also from maltose, and it secretes diastase.

Schattenfroh and Grassberger examined a long series of species, both pathogenic and non-pathogenic, and found that the latter consisted chiefly of two species, one of which is motionless, and is very widely distributed. It forms both short and long rods, particularly on alkaline substrata containing starch. It exhibits the granulose reaction in the *Clostridium* form (this usually disappears with the formation of spores), and it liquefies gelatine. The other species is motile, and forms thin rods with from six to twenty cilia on each. In the spore stage they are motile, and they do not liquefy gelatine. These two species include many of those previously described, which may be regarded as varieties. Neither of them attacks cellulose. We must here recall *Paraplectrum foetidum* (Weigmann), which is widely distributed in milk. It coagulates the milk, and then dissolves the coagulated mass, and develops a very objectionable smell of cheese.

There is no doubt that butyric acid fermentation may take place both in breweries, distilleries, and yeast factories, as well as in the fermentation of wine, which is probably caused by the activity of certain species. Thus butyric acid has been detected in potato fusel oil and in cognac, as well as in the yeast "goods" of the distillery, but more exact information is lacking.

If the preparation of the mash and wort goes on under indifferent conditions, a good opportunity is afforded for the development of such bacteria, and this applies also to the higher temperatures at which top fermentation is carried on.

A bacterium that produced butyric acid together with other substances is *Bac. lupuliperda*, described by Behrens, which occurs frequently on hops. The spontaneous heating of hops has been shown by Behrens to be due to the development of this and other organisms. It consists of motile cocci and short bacilli which liquefy gelatine. In nutrients free from saccharose it produces large quantities of ammonium compounds, and, in particular, trimethylamine (the smell of rotten herrings). In presence of saccharose the nutrient solution soon turns sour, and butyric acid is formed. The species appears to have its chief habitat in the earth, and bears much resemblance to *Bac. fluorescens putidus*, described by Flügge.

The sulphuring of hops appears to protect them particularly from moulds. To ensure that micro-organisms do not develop on soured hops the amount of moisture must not exceed 8 to 10 per cent., and they must be stored in a cold place.

#### 4. Bacteria Fermenting Cellulose.

These bacteria are just as widely distributed in nature as butyric acid bacteria. These are the organisms that ferment cellulose in plant residues present in the mud of rivers and ponds, and thus give rise to the evolution of marsh gas. It is only quite recently, however, that, thanks to the admirable researches of Omelianski, we have obtained a clear conception of what bacteria are responsible for this action. He sowed horse dung and river mud on Swedish filter paper (pure cellulose), with the addition of 1 gramm of chalk, 1 gramm of

potassium phosphate, 0.5 gramme of magnesium sulphate, 1 gramme of ammonium sulphate or phosphate, and a trace of sodium chloride to 1 litre of water. The fermentation was carried on at 34°-35° C. in flasks adapted for the cultivation of anaërobic bacteria. He thus proved that two different fermentations of cellulose are set up, a hydrogen ferment and a methane ferment, and that these are produced by two different species of bacteria. Omelianski separated the two by heating the fermenting material for fifteen minutes to 75° C. The hydrogen fermentation then proceeded, whilst before warming the methane fermentation took place. The reason is that the spores of the methane bacteria develop more rapidly than those of the hydrogen bacteria. If the liquid is heated to 75° C. after the germination of the spores of the methane bacteria, the vegetative rods of these bacteria will be killed, and only the spores of the hydrogen bacteria will remain alive and germinate. By repeated infection a microscopically pure growth of one or other species may be obtained.

The cause of the hydrogen fermentation is a thin bacillus, straight or slightly curved, which forms spherical spores at one swollen end. It is not coloured blue by iodine. The fermentation products consist of fatty acids, carbon dioxide, and hydrogen. The exciter of methane fermentation presents a similar microscopic appearance, but the threads are thinner and the spores smaller. It is not coloured blue by iodine. Its fermentation products consist about half of fatty acids (butyric and acetic acids) and half of carbon dioxide and methane. Other bacteria, and amongst them aërobic species, may ferment cellulose, and even moulds—e.g., *Botrytis* and *Cladosporium*.

##### 5. Alcohol-forming Bacteria.

Quite a number of bacteria produce alcohol amongst their products of fermentation. The first known species was discovered by Fitz in a cold extract of hay, and was afterwards more exactly investigated by H. Buchner, and described as *Bac. Fitzianus* (Fig. 26). It occurs both in coccus and bacillus forms. In a nutrient solution containing glycerine it ferments the

latter, forming principally ethyl alcohol. *Bac. ethaceticus*, discovered by P. Frankland in sheep manure, produces ethyl alcohol and acetic acid from glycerine, starch, saccharose, lactose, glucose, mannite, and arabinose. *Bac. pneumoniae*, described by Friedländer, is not only a pathogenic organism, but also has the power of decomposing saccharine nutritive solutions, and forming ethyl alcohol and acetic acid. In this connection may be mentioned a lactic acid bacterium found by Kruis and Rayman in sour yeast "goods" which produced ethyl alcohol as a by-product. Duclaux's *Amylobacter ethylicus*

has certain characteristics in common with *A. butylicus*, and occurs along with the latter, but produces ethyl alcohol and acetic acid.

Fitz found a species (*Bac. butylicus*) in cow dung which produces considerable quantities of butyl alcohol by fermentation of glycerine. Fitz describes it as occurring in the form of motile rods 5 to 6  $\mu$  in length and 2  $\mu$

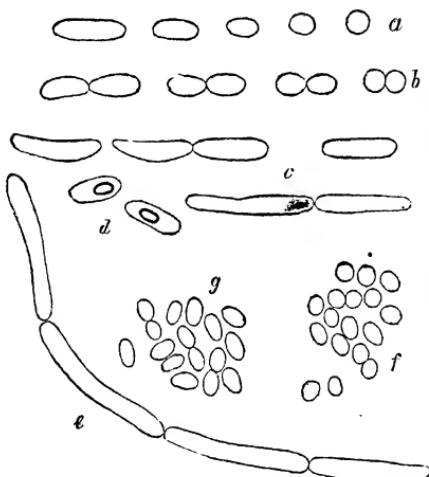


Fig. 26.—*Bacillus Fitzianus*, after H. Buchner.—a, b, f, g, *Coccus* forms and short rods; c, e, long rods; d, spore-bearing rods.

wide. He developed it in a solution containing 1 part of potassium phosphate, 0.5 of magnesium phosphate, 2 of pepsin, and 100 of glycerine in 2,000 of water, to which must be added 20 parts of calcium carbonate, and he found about 8 percent. of butyl alcohol in the fermented liquid. *Bac. orthobutylicus*, Grimbart, already described, also has the power of producing a considerable quantity of butyl alcohol, especially from glucose, when the nutritive liquid has an acid reaction, or when for any other reason the bacteria are in a feeble state. In the same way Perdrix's *Bacille amylozyme* yields this alcohol on fermentation.

Beijerinck's genus, *Granulobacter*, includes a series of bacteria producing butyl alcohol. We shall only attempt to describe a process used by him for the preparation of such species. He introduces coarsely ground meal of husked corn, in successive portions, into boiling water until the mass has the consistency of a thick paste. The last addition should not be subjected to a temperature of 100° for more than a few seconds. After rapid cooling, it is placed in an incubator at 35°-37° C. The pure cultivation may be carried out in sweet-wort gelatine under anaërobic conditions. The predominant species form white non-liquefying colonies, with *Clostridium* forms and oval spores.

Duclaux describes a facultative anaërobe, *Amylobacter butylicus*, obtained by infecting a potato mash with garden soil. It exhibits the usual swollen sporogenous cells and the granulose reaction. It ferments starch, and produces butyl alcohol, butyric acid, and acetic acid. A large amount of alcohol is readily produced when calcium carbonate is used to neutralise the acid formed during the fermentation of starch. The same alcohol is produced by fermentation of saccharose (which is not inverted), maltose, lactose, glycerine, mannite, and calcium carbonate.

Bacteria also occur which produce amyl alcohol (fusel oil); to these belong Perdrix's *Bacille amylozyme*, producing minute quantities of this alcohol from potato starch. A similar species was discovered by Pereire and Guignard, and H. Pringsheim isolated another from potatoes. It is still an open question how far the amyl alcohol produced during an impure alcohol fermentation is due entirely to the action of such bacteria. According to Ehrlich's experiments, fusel oil may be obtained by the action of alcohol yeasts on two of the decomposition products of albumen; leucin and isoleucin.

#### 6. Kephir, Koumiss, Mazun, Leben, Yoghourt, Ginger-beer.

Kephir, on which the investigations of Kern have thrown some light, is an effervescent, alcoholic sour milk, prepared by the inhabitants of the Caucasus from cows', goats', or sheep's milk. It is made by adding a peculiar ferment,

“kephir-grains,” to milk. These are white or yellowish and irregularly-shaped grains, not larger than a walnut and of a tough gelatinous consistency, and when dried become cartilaginous and brittle. The essential part of these grains consists of rod-like bacteria, connected in threads, and enveloped in gelatinous membranes. Kern calls this bacterium *Dispora Caucasicica*. According to Beijerinck this species, which he calls *Lactobacillus caucasicus*, produces in lactose, saccharose, glucose, and maltose a direct lactic acid fermentation. It produces solid, nodular colonies on whey gelatine resembling the kephir-grains. Besides bacteria, various yeast fungi and, frequently, moulds occur in the kephir-grains.

In the preparation of kephir a little milk is first poured on the grains and allowed to stand for twenty-four hours at about 17° C.; the milk is then poured off, and the grains preserved for future use. This milk is mixed with fresh milk, and poured into closed bottles, or leather sacks; the fermentation is completed in two or three days if the liquid is frequently shaken. It now contains about 2 per cent. of alcohol. This result is probably brought about by the simultaneous action of *Dispora* and yeast cells in combination with lactic acid bacteria present in milk. These ferments convert a portion of the lactose into lactic acid; the alcohol and a part of the carbon dioxide result from the action of yeast. As the fermented milk, according to some authorities, contains less coagulated casein than ordinary sour milk, it may be assumed that the *Dispora* is also able to partly liquefy (peptonise) the coagulated casein, perhaps with the help of the gelatinous mass secreted by the bacterium, found in the kephir-grains, but not present in the fermenting milk. According to recent investigations of Hammarsten, however, the amount of casein does not appear to decrease, but a part of it undergoes certain alterations, partly physical, in consequence of which it becomes more finely flocculent. The want of agreement in these results may possibly originate in the different biological composition of the selected kephir-grains.

Freudenreich regularly found *Dispora Caucasicica* (*Bac. Caucasicus*) in a number of kephir samples, which readily developed on milk-agar plates and in lactose broth at 35° C.;

the bacilli frequently have glistening points at both ends, and Freudenreich assumes that this phenomenon coincides with what Kern regarded as spores; unmistakable spores, however, were never found.

Two lactic acid coccus forms and a yeast species also occur in all samples. One of the cocci (*Streptococcus a*) forms diplococci and chains, and produces in lactose gelatine large, white colonies, with coarse granulation at the edge: the best temperature for the growth of this species is about 22° C.; it coagulates milk most rapidly at 35° C., and contributes essentially to the production of a sourish taste and fine flocculent appearance. The other coccus (*Streptococcus b*), likewise forming diplococci and chains, occurs in smaller colonies than *a*, and, in contrast with the latter, grows well at higher temperatures, and forms more acid than *a*, but does not coagulate milk. If this species is transferred, together with the kephir-yeast, to lactose broth, the fermentation is more vigorous than if the bacteria alone are inoculated; Freudenreich, therefore, presumes that *Streptococcus b* splits up lactose, and that its fermentation is rendered possible by the kephir-yeast. The *kephir-yeast* (a *Torula*) discovered by him grows remarkably well, and gives a weak fermentation in beer-wort, but does not appear to produce any fermentation in milk or lactose broth. The growth consists of oval cells (3 to 5  $\mu$  long, 2 to 3  $\mu$  broad); it forms neither film nor spores, and its optimum temperature lies at 22° C.

In the course of his experiments, Freudenreich succeeded in producing a liquor resembling kephir, for which purpose he inoculated a mixture of the four species in milk, and, after a lapse of some days, introduced a small portion of this sour, coagulated milk, which had been repeatedly shaken, into sterilised milk; he, therefore, concludes that these four species, through their symbiosis, are able to bring about the kephir-fermentation. He could not observe any synthesis of kephir-grains, and it is not yet clear what part *Dispora Caucasica* plays in the whole process; moreover, it appears to be highly probable that species of bacteria, other than the two coccus forms described by Freudenreich, in addition to other budding fungi, are active in the process. It may be

deserving of notice that in the author's laboratory it has been proved that a genuine *Saccharomyces* (*S. fragilis*) occurs in Russian kephir-grains which ferments milk-sugar independently, whereas all previous investigators only found budding fungi incapable of spore-formation.

In some parts of North America a ferment resembling kephir-grains is used in the fermentation of saccharine liquids. According to Mix' researches it contains a yeast-species which coincides with the one described by Beijerinck, and also a *Bacterium* which resembles Kern's *Dispora*.

If one of the kephir-grains is allowed to remain in milk, it grows very slowly, and only attains to double its size, according to de Bary, after the lapse of several weeks. He considers it probable that under such conditions single *Dispora* cells separate and give rise to new kephir-grains.

According to A. Levy's published process, kephir can also be obtained without the addition of Kern's ferments. When milk, which is turning sour, is repeatedly and violently shaken, an effervescent alcoholic kephir-like drink is obtained, which does not perceptibly differ from kephir prepared with kephir-grains as regards taste.

**Koumiss** is a similar fermented milk, prepared chiefly from mare's milk by the nomadic tribes of Southern Russia and Siberia ; it has been applied in many countries as a cure for various diseases. The true Koumiss, as prepared by the nomads, is fermented in leathern bottles, fermentation being started by adding a little dried milk from a previous fermentation. The organisms present sour and coagulate the milk during their development, and an alcoholic fermentation sets in, with evolution of gas. The coagulated mass is so finely divided that the liquid only turns thick. An accurate examination of the active organisms was undertaken by Schipin, who proved the constant presence of a yeast species, a lactic acid bacterium, and a special species of bacteria which occurred in large quantities, and appears to be characteristic of the Koumiss fermentation. It is a facultative anaërobe which forms whitish colonies in gelatine, consisting of a central nucleus with streamers in all directions. It thrives best on sour-milk gelatine, and does not liquefy the gelatine. By

the addition of cow's milk at 37° C. it coagulates to a thick paste without noticeable separation of whey. Its optimum lies between 20° and 30° C. Ten minutes' heating at 60° C. is sufficient to kill it. In experiments with mare's milk in presence of these three organisms, Schipin arrived at the conclusion that this species plays the most important part in the formation of Koumiss, and that it produces a lactic acid, as well as an alcoholic fermentation. It only displays its activity when the yeast and lactic acid bacteria have prepared the way for its development. At certain health resorts cow's milk is used instead of mare's milk, with the addition of sugar and alcohol yeast; in other words, a preparation which has nothing at all to do with Koumiss.

**Mazun** is, like kephir, a fermented milk (buffalo, goat, or cow's milk), which is prepared in Armenia, and is used both as a beverage and for butter-making. According to Kalanthal, Emmerling, and Lindner it contains a number of organisms, and amongst them yeasts fermenting lactose, an *Anomalus* form, *Bac. subtilis*, and lactic acid bacteria. In a similar way in Egypt, a sourish aromatic product resembling kephir is prepared from buffalo's, goat's, and cow's milk named **Leben**. It contains less alcohol than kephir, and coagulates in an alkaline mass. As in previous cases, boiled milk is brought into fermentation by the addition of dried milk from a previous fermentation. According to Rist and Khoury five different species are active in this fermentation: a *Streptobacillus* which coagulates milk and produces lactic acid from lactose; a very thin *Bacillus* which also yields lactic acid; a *Diplococcus* which strongly coagulates milk; a yeast species which ferments glucose, saccharose, and maltose, but not lactose, but which, along with the *Streptococcus*, may give a vigorous fermentation in milk, as the bacterium hydrolyses lactose; and, finally, a *Mycoderma* species which can ferment glucose and maltose, but not lactose. The *Streptococcus* and the *Diplococcus* also possess a special coagulating enzyme. According to Rist and Khoury, by the use of these five species, Leben can be prepared from milk, and, best of all, if the two budding fungi are added first and the bacteria later.

**Yoghourt** is a species of sour milk or thick milk prepared in Turkey and Bulgaria. Sheep's or cow's milk is used, which is boiled and reduced by evaporation to half its volume, then cooled to 45° C., and the ferment—"Maya" or "Podkoassa"—is added. This consists of milk residues from previous preparations, dried under special conditions and ground, and contains many species of bacteria. After a fermentation lasting for nine to sixteen hours at a temperature of 40° C., the Yoghourt is ready for consumption. It is more or less solid, according to the degree of concentration, and possesses a sourish aromatic taste. It is eaten cold, either alone or with the addition of rice, bread, sugar, or fruit syrup. We owe the first bacteriological investigation to Grigoroff, who found three different lactic acid bacteria. The most important is *Bacillus A.* (*Bac. Bulgarus* or *Bulgaricus*). It forms long motionless rods, often linked in chains, grows well on saccharine substrata, has an optimum température of 45° C., and does not multiply at room temperature. It produces alcohol, and attacks lactose, mannite, dextrose, maltose, and lœvulose, but not rhamnose, dulcite, and sorbite. *Micrococcus B.* occurs as single cocci or diplococci. In addition to the above varieties of sugar, it attacks rhamnose and glycerine. *Streptobac. C.* forms short rods linked in chains. It attacks lactose, saccharose, lœvulose, and glycerine, but not maltose, mannite, rhamnose, dulcite, or sorbite. The optimum temperature for the last two species is 45° C., and they produce alcohol. Other investigators (Mazé) have only found two species of bacteria. Luerssen and Kühn, as well as Kunze, mention a "granule bacillus," perhaps a variety of *Bac. Bulgaricus*. Others have found yeast species to which they attach more or less importance. Metschnikoff, Piorkowski, and Henneberg have published further work regarding Yoghourt.

The **Ginger-beer Plant**, which presents morphological resemblances to the kephir ferment, has been examined both botanically and biologically by Professor Marshall Ward. If this ferment is introduced into saccharine solutions containing ginger, it transforms them into an acid, effervescing beverage, ginger-beer. When fresh, it forms solid, white, translucent lumps, of irregular shape, brittle like dried jelly, varying in

size from that of a pin's head to that of a large plum. It induces an alcoholic fermentation in the sugar solution, which at the same time becomes viscous. Marshall Ward isolated the numerous micro-organisms existing in these lumps, and described a series of yeast-fungi, bacteria, and moulds, and of these, two organisms proved to be essentially concerned in the fermentation of ginger-beer. One is a *Saccharomyces* (bottom yeast), belonging to the ellipsoidal group of this genus, and probably originating from the ginger and brown sugar commonly used; Ward named it *Saccharomyces pyriformis*. It inverts cane-sugar, actively ferments the products, and forms a pasty white deposit at the bottom of the vessel. It yields spores on gypsum blocks in 40 to 50 hours at 25° C.; it also forms spores on gelatine. In hopped wort it induces a feeble fermentation, and forms a film on the surface containing many pear- and sausage-shaped cells.

The other essential organism, which is always present, is a Schizomycete, *Bacterium vermiciforme*, which, according to Professor Ward, emanates from ginger, and is active in the lactic acid fermentation. It is a peculiarly vermiform organism, enclosed in clear, swollen, gelatinous sheaths, and imprisoning the yeast cells in brain-like masses formed by its convolutions. It is the swollen sheaths of this organism which constitute the jelly-like matrix of the "plant." It also appears without sheaths, and in a great variety of shapes. The gelatinous sheaths are only developed when the saccharine liquid is acid, and free from oxygen.

A *Mycoderma* and a *Bacterium aceti* were also found.

Marshall Ward has proved experimentally that *Saccharomyces pyriformis* and *Bacterium vermiciforme* are the only two essential species in the ginger-beer fermentation, since it was only by inducing a fermentation with these two species that he was able to produce an effect similar to that obtained when the ordinary ginger-beer plant is employed. But it is only when both species develop together in the liquid that they bring about this result, and his experiments indicate that the relations between the yeast and the bacterium are those of true symbiosis, because the yeast ferments more vigorously in presence of the bacterium than it does alone.

### 7. Slime-forming Bacteria.

Among the various species of slime-forming bacteria there are several which are of peculiar interest in the fermentation industries, as they occur in wine, milk, beet juice, and fermenting wort, causing morbid changes. By analogy, this slime formation, which usually consists of substances resembling gum, may be regarded as a phenomenon closely related to the commonly occurring zoogloea formation of certain bacteria.

In his *Études sur la bière* (Plate 1, Fig. 4) Pasteur described bead-like chains of spherical organisms, which render wine, beer, and wort so viscous that they can be drawn out into threads ; this is caused by the formation of gum and mannite.

Kramer has described *Bacillus viscosus sacchari*, which in a short time converts neutral or slightly alkaline cane-sugar solution into a tough mass of a gummy nature. He isolated a *Bac. viscosus vini* (2 to 6  $\mu$  long), which was cultivated in sterile wine, air being excluded. Sound wines infected with this growth thickened in the course of six to eight weeks. It grows best at 15°-18° C., and apparently cannot exist at such a comparatively low temperature as 30° C.

A mannite fermentation is sometimes associated with the formation of slime in wine. The motionless bacterium isolated by Gayon and Dubourg grows on the bottom as large zoogloea, and thrives only in saccharine solutions. On the other hand, a mannite fermentation investigated by Peglion in wine is never accompanied by the formation of slime.

A very comprehensive memoir regarding slime-forming bacteria has been published by Kayser and Manceau. The disease occurs especially in wines protected from the action of the atmosphere. The formation of slime begins in the lowest stratum of the liquid, and increases by degrees without reaching the top. The disease occurs in those districts where it is customary to remove the skins, stones, and stalks, before fermenting the juice. Such bright wines are usually poor in tannic acid, nevertheless other wines comparatively rich in tannin may be attacked. The percentage of alcohol and of free acid appears to be of greater importance than the content of tannic acid.

Bottled wines suffer more than wine in the cask; white wines more than red, because the former contain more sugar, especially lactulose, which forms an excellent food-stuff for these fermentations.

Slime-forming bacteria have been isolated from eight different wines, some white and some red, and six of the species were subjected to an exact investigation. They all occur as short rods, varying in length and breadth (1.1 to 4.2  $\mu$  long, 0.7 to 1.7  $\mu$  wide). They seldom occur singly, usually linked in chains, which are short and straight in some cases, and in others long and spiral. They are non-motile, anaerobic, neither form spores nor liquefy gelatine, and all give the Gram coloration. They are surrounded by a slimy growth at every stage of development. The optimum temperature for propagation lies between 25° and 30° C.

Bacteria have been found in bread which produce a strong formation of slime, and in particular the "potato bacilli" appear to be active—i.e., varieties of *Bac. mesentericus vulgatus* (*Bac. panis viscosi*), described by Kratschmer and Niemitowicz, and by Uffelmann, Thomann, Vogel, and others. As a consequence of the action of these bacteria the bread can be drawn out into long thin glutinous strings. They occur in rye meal and multiply in presence of moisture. They develop in bread if the spores survive the baking temperature, and the bread is stored in a warm place. According to Migula, *Bac. panis* (Vogel) occurs in long slender rods (4 to 7  $\mu$ ), forming chains, which have a rapid movement, and possess a polar cilium. They form oval spores, which survive the action of a current of steam at 100° C. for fifteen minutes (in a potato culture). On gelatine plates the colonies form flat liquefied depressions. With a magnification of 70, it appears as a colony having a yellowish-brown nucleus coarsely granulated, and delicate streamers in the gelatine. On agar also the colonies form a nucleus with streamers. The optimum is at 40°-42° C.

In plant infusion (*digitalis* leaves), Ritsert proved experimentally the presence of a *Bact. gummosum* which brings about a mucilaginous formation of slime. Its activity depends upon the sugar content of the liquid, and is greatly favoured by the presence of potassium and sodium acetate and yeast ash.

There is a rich formation of slime in 10 to 30 per cent. nutrient sugar solution, whereas none occurs in similar grape-sugar and milk-sugar solutions. The species has a pronounced demand for oxygen, and the cells exhibit movement at certain stages. It appears to occur both as rods and coccus forms, according to the composition and reaction of the substratum. It liquefies alkaline gelatine. In a stab-culture on agar it grows as a moist glistening whitish deposit, which forms two zones, the inner wrinkled and the outer smooth. Bräutigam isolated a *Micrococcus* from an infusion of *digitalis* leaves, which converted a nutrient sugar solution into a complete jelly, and made apple juice viscous. In a similar infusion Happ found a slime-forming rod bacterium (*Bac. gummosus*) 5 to  $7.5\text{ }\mu$  long, 0.6 to  $2\text{ }\mu$  wide. It assumes spindle shapes in old cultures, and is sometimes motile. On neutral gelatine it forms colonies with streamers; the gelatine is liquefied. On potatoes it forms coccus-like involution forms. Saccharose solution is absolutely necessary for the production of slime. The optimum lies at  $25^{\circ}$ - $30^{\circ}$  C. He also found a *Micrococcus gummosus*, which may be distinguished from Bräutigam's species by its fermentation products. It forms yellowish colonies on gelatine, but a colourless deposit on agar. The optimum is at  $15^{\circ}$ - $20^{\circ}$  C. This species may produce slime in saccharose and maltose solution. Schardinger has undertaken a detailed enquiry into the products formed by a slime bacterium, one species of which was isolated from impure drinking water. It is a very short, motionless, non-sporogenous bacterium which forms on gelatine a tough slimy film consisting of cells linked in long chains. On saccharose or grape-sugar gelatine it forms slimy and "ropy" colonies of a greyish-white appearance, which when removed leave a depression in the gelatine. In broth it forms slimy flakes, especially on the surface, and it also makes milk viscous. In nutritive liquids containing saccharose, maltose, lactose, etc., it causes fermentation with evolution of hydrogen, and by fermentation of an 8 per cent. saccharose solution, with inorganic salts and calcium carbonate to neutralise the acid, it forms lactic acid, acetic acid, ethyl alcohol, and succinic acid. The optimum for slime formation is  $20^{\circ}$ - $30^{\circ}$  C. It does not liquefy

gelatine. According to Schardinger, the species is related to Löffler's *Bac. lactis pituitosi*. A chemical examination of the slime formed by mass cultures from saccharose solutions, containing nutritive salts and calcium carbonate, shows that it chiefly consists of a carbohydrate which by oxidation with nitric acid forms mucic acid, and by boiling with hydrochloric acid produces optically active sugar. As a slime can also be formed by bacteria in the absence of sugar, it should probably be regarded as a product of the swelling of bacterial membrane.

As an example of one of the species producing a vigorous formation of slime in milk may be mentioned *Bac. lactis viscosus*, found in water, and described by Adametz. It forms a short, feebly-motile rod with a thick refractive capsule. Its average dimensions (in milk cultures) are  $1\cdot5\ \mu$  long and  $1\cdot25\ \mu$  thick. On glycerine-peptone-gelatine it forms whitish non-liquefying colonies with irregular jagged edges, which shows a bright opalescence in reflected light. By inoculation in sterilised milk, the milk becomes viscid like honey in four to six weeks, and may be drawn out into long threads. At the same time the fat globules of the milk disappear. Lactose is only attacked to a very slight extent by this species, whereas casein is greatly modified. Slime is also formed in nutritive liquids free from carbohydrates. It is believed to be a zoogloea formation.

Other related species have been described by Duclaux, Leichmann, Schmidt-Mühlheim, Löffler (*Bac. lact. pituitosi*, motionless rods which quickly divide into coccus-like cells, and on gelatine give white colonies with sharp or slightly dented edges), Weigmann (the coccus of "lange Wei" with nitrogenous slime), Emmerling, etc.

Emmerling has proved that *Bac. lactis aërogenes* forms a mucilage in lactose solutions possessing the properties of galactan, for by oxidation it may be transformed into mucic acid.

In beer also slime-forming bacteria occur. Thus H. Schröder (1885) found a *Micrococcus* in "ropy" Berlin "Weissbier," which was afterwards cultivated in a pure state by P. Lindner, who named it *Pediococcus viscosus*. The disease

could be produced by adding pure cultures to sterilised "Weissbier" wort. On the other hand, this organism had no action on hopped beer-wort or low-fermentation beers. By the addition of tartaric acid the beer becomes normal. Schönfeld distinguished many species in long "Weissbier," and, in particular, found two typical kinds (*P. major* and *minor*). The optimum for the formation of slime lies between 20° and 26° C. These species form a considerable amount of acid, and impart to the beer a pleasant, acid-wine bouquet. In presence of larger quantities of alcohol the beer does not easily turn viscid, and the lactic acid present protects such beer from the disease.

These organisms grow well, according to Schönfeld, in ammoniacal yeast decoction. Schönfeld has proved that such species occur in horse urine.

Inropy Belgian beer, Van Laer found the cause of this disease to be small and very thin, sporogenous rods (1.6 to 2.4  $\mu$  long), which were partly isolated and partly united in pairs by means of a zooglœa-like substance. When added to beer-wort, this first becomes turbid, and afterwards ropy. Milk also turns slimy, and its lactose ferments. On beef-broth gelatine these rods give concave colonies with concentric rings of different colours; streak cultures give broad, white bands, with a sinuous border; stab-cultures give a white stripe soon extending to the bottom of the glass; the gelatine forms fissures which become filled with the growth, while at the same time a speck is formed on the surface. Experiments carried out with pure cultures of this bacterium in beer-wort have shown that one and the same form includes many varieties, which have a somewhat different action on wort. They are all included under the name *Bacillus viscosus* (I. and II.). If sterilised wort is infected with this bacterium, and alcoholic yeast added after the lapse of some hours, the liquid becomes viscous. If the wort is infected with a mixture of absolutely pure yeast and bacteria, the disease will develop in a varying degree, according to the proportion of bacteria. If, however, these are only added after the completion of the primary fermentation, the disease will not appear at all. The greater the proportion of nitrogenous matter in the liquid, the sooner it will become viscous; even liquids which do not contain sugar

can be maderopy by these species. When the nutritive liquid contains much sugar, the fungus develops very feebly, and in pure sugar solutions the phenomenon does not occur. A high content of acid greatly restricts the development of these bacteria.

Van Laer has since isolated a *Bac. viscosus bruxellensis* which produces, in addition to slime, a peculiar disease called "bière à double face." It occurs in "spontaneously" fermented Belgian beers, Lambic, Faro, and Mars, and can be recognised by the fact that the beer looks clear in transmitted light, and milky in reflected light. It forms a long rod making a white tough film on beer-wort, which grows down into the liquid. Subsequently the slime disappears, and the rods are then surrounded by a slimy envelope. On wort gelatine large, round, slimy, transparent colonies are formed, with a yellow centre and with many zones. The species restricts the activity of alcohol yeasts, and beer attacked by it is consequently poor in alcohol, and richer in extract than sound beer. It forms lactic, acetic, and butyric acids.

Vandam found in English beers an aërobic *Bac. viscosus* (III.), which occurs as small rods, single or in chains, consisting of two, three, or more links, with spore-formations in the centre of the rods. This bacillus develops best at about 30° C., and produces a slimy mass in brewers' wort, which under the microscope proves to consist of zooglœa formation. After the lapse of some time the liquid has the consistency of albumen. No gas is evolved, but the liquid acquires a peculiar odour. On meat-juice gelatine and on wort-gelatine the growth develops freely. The viscosity of the liquid does not seem to depend on the quantity of nitrogenous matter present, but on the other hand, the bacillus grows feebly in the absence of sugar. This species is incapable of producing disease in beer unless it is thriving well, and is introduced in large quantities into the wort before or during pitching. Like the form discovered by van Laer, it ferments milk-sugar; and, according to Vandam, it is easy to detect it in yeast, even in traces, simply by introducing a sample of the latter into nutritive liquid containing milk-sugar, a growth of this species soon making its appearance in the upper part of the liquid.

Brown and Morris mention a *Coccus* form which also seems

to produce ropiness in English beer. This species occurs as diplococci and tetrads, and gives yellow wax-like colonies on meat-juice gelatine. The disease made its appearance in the beer after a lapse of six to eight weeks, but it was not usually possible to produce it by inoculation with pure cultures of the species in sterile beer. Close to the fermentation room there was a pork-butcher's premises, in which putrefying matter had accumulated ; after this had been removed and the soil dug and cleaned, the disease disappeared.

Fellowes also examined several English beers affected by this disease, and prepared pure cultures of the bacteria present, but by inoculation of the cultures in beer he did not succeed in preparing a beer containing these organisms and showing a viscosity corresponding to that of the sample from which they came.

Heron undertook a thorough study of a slime-ferment which occurred in English beers, a very small coccus, which gradually elongates, and by contracting in the middle assumes the form of a dumb-bell. The two ends may also expand in a direction at right angles to the first growth, and assume a similar shape. At a later stage the species takes on the form of rosaries (*zooglœa*). The beer attacked loses its acid simultaneously with the formation of mucilage, and acquires an unpleasant taste. This species can only produce slime in presence of yeast. Beer may be protected against its action by increasing its acidity and adding more hops. The species originates in malt dust, according to Heron.

The so-called frog-spawn fungus *Leuconostoc (Streptococcus) mesenterioides* was investigated by Cienkowski and van Tieghem and subsequently by Zopf and Liesenberg (Fig. 27). Both the European form and the variety found by Winter in Java occur spontaneously in beet-juice and in the molasses of the sugar factory, and in molasses distilleries, in which they form large slimy masses ("frog-spawn") and multiply vigorously. The fungus forms chains of cocci, alternate pairs of which are always more closely united. In contrast to the observations of earlier workers, who thought that certain of these cocci enclosed spores, Zopf found that they present no differences morphologically or physiologically ; spore-formation could in

no case be proved. Consequently, the analogy formerly assumed to exist between this fungus and the algæ genus *Nostoc* (implied in the name *Leuconostoc*) falls through.

Under certain conditions the cells are surrounded by a strong gelatinous sheath with a sharp outline (*Bb*, *Bc*, *C*), which in many of the above consists of a mucilaginous carbohydrate, *dextran*. This formation only takes place in the

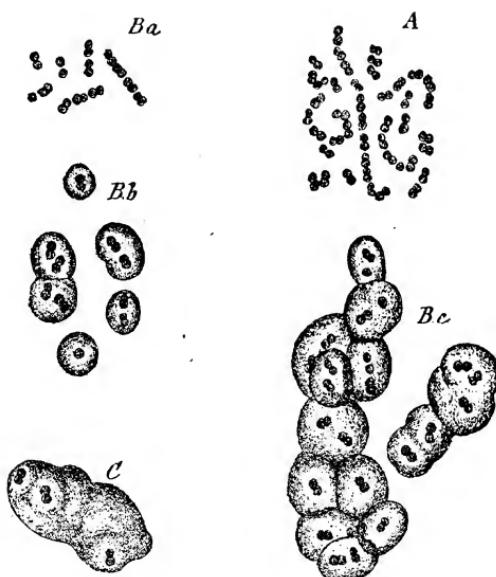


Fig. 27.—*Leuconostoc mesenteroides*, Cienkowski (after Zopf).—*A*, Cell cluster of the sheathless variety, taken from a potato cultivation; *B*, series showing the development of a culture, grown in gelatine, free from sugar; *Ba*, sheathless; *Bb*, the same after 24 hours' growth in a solution of molasses, sheaths already seen but not strongly developed; *Bc*, after 48 hours' growth in molasses, the sheaths strongly developed and partly encased in each other; *C*, a small gelatinous mass from which the cells have been expelled.

presence of cane and grape-sugar, and not in solutions of milk-sugar, maltose, or dextrin. Under the latter conditions, and in potato cultures, the species develop distinctive forms, in which the gelatinous sheath is completely absent (*A*, *Ba*). The formation of jelly is a phenomenon depending also upon certain conditions of nutriment.

*Leuconostoc* ferments grape-sugar, cane-sugar (after previous

inversion), milk-sugar, maltose, and dextrin, with production of acid and gas. The acid proved to be lactic acid. Especially characteristic of this fungus is its power of resisting high temperatures, the younger growths possessing this power in a higher degree than older cultures. It withstands gradual heating to 86°-87° C. for a few minutes. The optimum temperature for development lies between 30° and 35° C.; the maximum at 40°-43° C. It is also remarkable that both the growth and the fermentative activity of the fungus are favourably affected by the presence of considerable quantities of calcium chloride.

Cohn's *Ascococcus* (*Micrococcus*) *Billrothi*, the cells of which are enveloped in a jelly, under certain conditions of nourishment, forms mucilaginous slime from sugar, according to Zopf. The three following species may be classed along with the above:—Glaser described a *Bact. gelatinosum betae* which produces slime in beet-juice and evolves gas. It forms short motile rods, giving white liquefying colonies on beet-juice gelatine. At its optimum of 40°-45° C., it rapidly forms a gelatinous film on beet-juice; it does not, however, develop on molasses. It inverts saccharose, and produces alcohol during fermentation. The slime is of the same character as in *Leuconostoc*. *Clostridium gelatinosum*, described by Laxa and Schöne, is found in sugar factories, and forms a slime like that in *Leuconostoc*. It appears as rods of varying length, which are motile in their earlier stages, and form spores in the middle of the swollen cells. The optimum is at 40° C. The species inverts saccharose, and thrives best with free access of air. In soil where sugar-beet is cultivated it grows in great numbers. Other species, both coccus and rod forms, are described by Schöne. Maassen has described a number of similar species under the general name of *Semiclostridium*, by which he wishes to express that the rods, especially when the quantity of oxygen is restricted, swell at one end and in the middle. The ellipsoidal spores do not, however, develop in this swelling, but at the thin end of the cell; the young rods are motile. The optimum for vegetative growth is about 45° C. The spores are extraordinarily resistant, both to boiling and to anti-septics, and the organisms are widely distributed in the soil.

*S. commune*, isolated from filter press residues, forms a

jelly only from saccharose, which is inverted by this species, and fermented with evolution of carbon dioxide. It may be distinguished from *Leuconostoc* by the fact that the slime yields levulose on hydrolysis, whilst *Leuconostoc* slime forms dextrose.

Cobb describes a gum disease on the sugar cane, causing the production of a slimy yellowish mass in the vascular bundles of the stem, filled with bacteria of a single species, *Bac. vasculorum*, which, according to Cobb, produces the mucilage. In the gummy runnings of the sugar cane, a short rod with cilia always occurs, according to Smith, who named it *Bact. Sacchari*. The "gummosis" of turnips and sugar beets, recognisable by drops of gum appearing on the cross sections, which acquire a black colour, is accompanied by a strong development of bacteria. These gradually multiply, and entirely alter the character of the mass. Busse (experiments on the inoculation of pure cultures into sound beets) proved that the short motile rod which forms slimy colonies both on gelatine and on slices of beet was the cause of the disease. It inverts saccharose.

#### 8. Bacteria with Inverting, Diastatic and Proteolytic Enzymes.

We have already mentioned a number of bacteria that owe their importance in the fermentation industry to enzymes. Some further examples are given in this section which possess other enzymes.

*Invertase* is present in the following, amongst others :—

*Bac. (Proteus) vulgaris*, one of the commonest putrefactive bacteria, forming short motile rods often grouped in rows, and also forming long filaments with spiral and spirulina forms.

*Bac. fluorescens liquefaciens*, which occurs frequently in water, as well as in decomposing substances, and derives its name from a greenish fluorescent colour which it imparts to gelatine. The gelatine is liquefied. It forms straight and curved rods of medium size, consisting of two or more members.

*Bac. Megatherium*, found by de Bary on boiled cabbage leaves, is distinguished by its extraordinary size. The rods may be  $2\cdot5 \mu$  thick; they sub-divide into short cells. It forms whitish, liquefying colonies on gelatine.

Fermi and Montesano found that *Bac. Megatherium*, *Proteus vulgaris*, and *Bac. fluorescens liquefaciens* in neutral broth, invert a 4 per cent. solution of saccharose. Many of these bacteria, however, lose their power of inversion if the broth

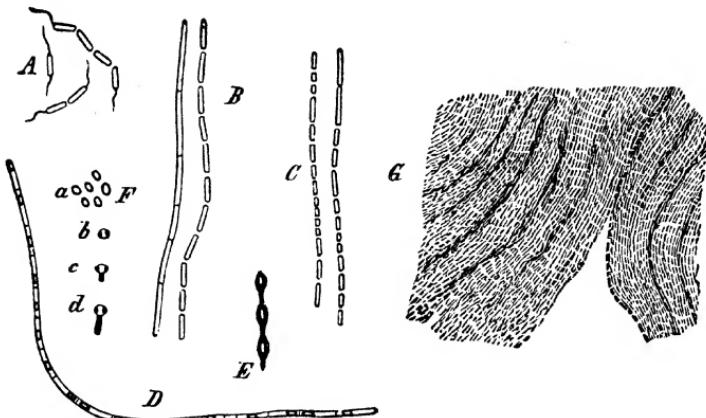


Fig. 28.—*Bacillus subtilis*.—A, Cells with cilia; B, C, segmented threads; D, thread with spores; E, spores in swollen mother-cell; F, germination of spores; G, film on hay infusion.

is rendered alkaline, whilst most of them are uninjured in slightly acid broth. In broth without sugar, and in media containing no albumen, such bacteria produce invertase; thus almost all the species that were examined formed invertase in a nutritive salt solution containing glycerine.

The invertase produced by these bacteria proves to be a soluble enzyme, which is destroyed at temperatures differing according to the species, but it is always more resistant during its action on saccharose than in a dissolved state; it is very sensitive to acids and alkalies, and especially to organic acids and potash.

According to A. J. Brown, the well-known *Bac. subtilis* (hay bacillus, Figs. 28 and 29), belongs to this group. Brown found that it cannot grow either in beer or wort of normal acidity.

According to Hansen, many species of bacteria of common occurrence in beer secrete inverting ferment. Amongst these

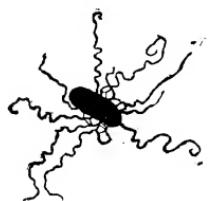


Fig. 29.—*Bacillus subtilis* (after A. Fischer). Cilia staining.  $\times 1500$ .

there is a group which exhibits an inverting action on a pure saccharose solution, but loses this property when yeast-water is added.

Wortmann in 1882 began some experiments on the **diastatic action** of bacteria, and used for this purpose drops of bacterial liquids on rotten beans or potatoes. He proved that in this mixture species were present which can bring about the same changes in starch paste and in soluble starch as the diastase of the higher plants can do. The bacteria only exert action on starch when no other available carbohydrate is present (e.g., sugar or tartaric acid). This was established by means of experiments with inorganic nutritive solutions. Krabbe showed that the presence of peptone increased the formation of diastase. Fermi proved that this enzyme was present in different *Streptothrix* species, and found that the formation of diastase was prevented when the bacteria were cultivated on substrata free from albuminoids. Pfeffer and Katz observed a rich formation of diastase in *Bac. Megatherium*; by the addition of saccharose or maltose to the nutrient the diastatic activity was considerably reduced. Garbowski observed the enzyme in his detailed researches on *Bac. luteus*. The action is brought out most strongly by inoculating on an inorganic nutritive liquid, together with starch solution.

*Bac. vulgaris* and *Bac. prodigiosus* are amongst the organisms containing **proteolytic** or **peptonising enzymes**. The latter, which belongs to the group of colour-forming bacteria (the "Bleeding Host") forms very short motile rods in weakly alkaline substratum, but longer rods and filaments in weak tartaric solutions. These albuminoid-digesting bacteria play an important part in nature, in the decomposition and successive building up of organic substances. The anaërobic species appear to be particularly active; for example, *Bac. putrificus*, which forms long motile rods, with spore-formation at their swollen ends.

#### 9. *Sarcina*.

The name *Sarcina* is given to the spherical bacteria (*Ooccus*), which are motionless, and divide in all three planes. Under favourable conditions of growth, and especially in liquids,

the cells formed by division may remain clumped together, caught in the slime secreted by the cells, and thus more or less cubical groups are constituted, which sometimes have a certain resemblance to corded bales of goods. On solid substrata, on the contrary, the growth of many of these species breaks down rapidly into single cells, or else remains grouped in clusters of two and four. The harmful kinds occurring in beer, which belong to this group of bacteria, are really only known with division in two planes, and the production of regular shapes (diplo- and tetracoccus), whilst the larger clusters are composed of irregularly massed cells. Until something further is known about them, these species must, therefore, be counted in the group which shows division in two planes (*Pediococcus*, *Micrococcus*, and *Merismopedia*). A similar behaviour is shown by *Sarcina rubra*, discovered in red milk by Menge, which in milk only displays the micrococcus form. The sarcina form is known in broth-cultures.

The many species of *Sarcina* that have been described can give differently coloured colonies on gelatine; white or greyish colonies are formed by *S. alutacea*, isolated by Gruber from leaven, which liquefies gelatine. This is also the case with Lindner's *S. candida*, found in the water reservoir of a brewery. Yellow colonies are formed by the widely distributed *S. flava* which has been found in leaven, beer, and elsewhere. It forms both regular packets and irregular masses of cells. On gelatine it gives small round colonies, which gradually liquefy the gelatine, and on hay infusion it forms a film with a strong development of packet shapes. *S. aurantiaca* forms on gelatine orange-yellow, liquefying colonies, but develops the typical sarcina form only in hay infusion and plant decoctions. It gives a dark bluish-green colouration with sulphuric acid. *S. casei*, discovered by Adametz in cheese, forms pale yellow, liquefying colonies with concentric rings, and coagulates milk. Adametz also found *S. butyrica* in cheese; it forms a yellowish-white colony on the surface of stab-cultures in potato gelatine, but dark liquefying colonies in plate-cultures. *S. lutea* always forms regular packet shapes, and gives on gelatine lemon-yellow, non- or only feebly liquefying colonies. Brown colonies on gelatine are given by *S. acidificans* discovered by

Adametz in cheese. It develops a yellow colony in stab-cultures on agar. It precipitates casein from milk. A dark brownish-yellow colour is developed by *S. fusca*, discovered by Gruber in flour. Among the varieties exhibiting a red growth are *S. rubra*, giving glistening red colonies on the surface of gelatine, and slowly liquefying it. The colouring matter is insoluble in alcohol. *S. rosacea*, occurring in air and water, usually forms irregular masses of cells in malt extract, but develops the typical shapes in a hay decoction, which are surrounded by a brownish envelope. On a neutral malt extract gelatine it forms a reddish deposit with a dry surface. The colouring matter is soluble in warm alcohol. *S. maxima*, found by Lindner, which develops in a malt mash at 40° to 45° C., has cells of 3 to 4  $\mu$  diameter.

*S. mobilis*, isolated by Wolff from milk, is distinguished from each of the above by having motile cells. It shows the typical form both in liquids and on solid substrata, liquefies gelatine, and forms yellow colonies on whey gelatine and agar.

In the fermentation industry, sarcina-like organisms occur in addition to those already mentioned in section 8, especially in low-fermentation lager beer, where they may develop during the secondary fermentation. Pasteur described and depicted the *diplococcus* and *tetracoccus* forms, and he noted that beer contaminated with such bacteria assumes a disagreeable flavour and smell. At a later date they were depicted by E. C. Hansen under the name *Sarcina* (Fig. 30). He found them in many parts of the brewery plant, and established by direct experiments the nature of their influence on beer. Balcke, who afterwards investigated such beer, regarded it as established that the sarcina-like organisms were responsible for the disease, and as he only found the *diplo-* and *tetracoccus*, and not the packet form, he gave it the name *Pediococcus cerevisiae*. Other workers have since failed to isolate the typical sarcina form from diseased beer, and have only detected irregularly massed cells, so grouped that it was impossible to determine how they were produced. The possibility is not, however, excluded that we may be able, as in other cases, to detect typical sarcina forms in these



Fig. 30.—Sarcina.

diseases. The name " *Sarcina* disease " is best retained, as certain conceptions are associated with it.\*

Lindner has described a number of *Sarcina* species in pure cultures, and amongst them one which occurs in diseased lager beer, which he named after Balcke, *Pediococcus cerevisiae*. It grows best with access of air, and forms yellowish or yellowish-brown colonies on meat-juice gelatine, which is not liquefied. In a stab-culture it gives a flat white colony on the surface. In the streak culture it forms a greyish-white moist line, which appears iridescent in thin layers. On meat-juice gelatine it is killed by eight minutes' exposure to 60° C., but not by twelve minutes' exposure at 50°-55° C. It proved impossible by inoculating pure cultures to reproduce the unpleasant flavour and odour of the beer; only turbidity ensued, and the isolated species doubtless cannot have been a disease *Sarcina*.

In later experiments Lindner succeeded in a few cases in reproducing the characteristic appearance in beer by the application of yeast which had been inoculated with a *Sarcina* isolated from the diseased beer. On the other hand, A. Petersen observed a case where a growth of these organisms had developed in beer without affecting either its flavour or its smell.

A. Reichard isolated from low-fermentation beer a *Pediococcus sarcinaformis*, which developed freely in sweet wort and sterile beer, but not in pasteurised beer. This species developed best when access of air was limited. In fermentation experiments turbidity or peculiar changes of taste occurred in certain cases, but not in the majority. After many experiments, he arrived at the conclusion that these contrary results were due partly to the condition of the various growths of this *Sarcina* form, partly also to the manner in which the fermentation took place. In cases of quiet fermentation in

\* As examples of the various typical *Pediococcus* = *Micrococcus* species, may be instanced *M. candidus* and *concentricus* growing in water; *M. ureæ*, which converts urine into ammonium carbonate, and *M. lacticus*, which Marpmann found in fresh milk, giving white and non-liquefying colonies; *M. amarificans*, which, according to Conn, makes milk bitter, and *M. casei amari*, which, according to Freudenberg, makes both milk and cheese bitter: these have white liquefying colonies; *M. luteus* with yellow non-liquefying, and *M. flavus* with yellow liquefying colonies; *M. cinnabarinum* and *carneus*, isolated from water, with red colonies.

a lager cask the growth remained at the bottom, and the bacteria did not exert any appreciable influence on the liquid, whereas in the case of a vigorous secondary fermentation they were carried upwards in the liquid, along with the carbon dioxide bubbles, after which the disease manifested itself. Rousing the beer might, therefore, be injurious in such cases. An addition of hops to lager beer exerts a retarding influence on these bacteria, as on the majority of bacteria occurring in beer.

Schönfeld inoculated cultures (from diseased beer cultivated on yeast-water gelatine) into pasteurised beer, and produced, not only a turbidity, but also an acid—sweet, disagreeable flavour. Two species have been described by N. H. Claussen, which were isolated after he had checked the growth of other organisms occurring in beer by a slight addition of acid ammonium fluoride. The beer-cultures were allowed to develop in hopped wort and in pasteurised beer, and when inoculated in beer brought about the characteristic disease phenomena. Both species grow in the usual nutritive liquids either neutral or slightly acid, whereas a minute quantity of free alkali restricts their growth. The most favourable temperature for their growth is 23°-24° C. Neither liquefies gelatine. They grow in wort both when oxygen is fully excluded, and also in presence of the full quantity in the atmosphere. The one, *P. damnosus*, usually imparts an unpleasant odour and flavour to beer, but only forms a slight deposit in the liquid ; the other, *P. perniciosus*, causes, in addition to the deterioration of flavour and odour, a turbidity in the liquid. Schönfeld has isolated species from diseased beers with the help of sweet wort gelatine, and especially on dry-yeast gelatine. He found that species that are dangerous for lager beer, imparting the *Sarcina* odour and objectionable flavour, as well as turbidity, only produce a comparatively minute quantity of acid in sweet wort, and give a peculiar odour slightly resembling honey : for this reason he gave the group the common name of *P. odoris mellisimilis* (he assumes that this group is identical with Claussen's *P. perniciosus*). In contrast with this a group of species exists which occur in lager beer and "Weissbier" producing large quantities of acid in sweet wort, and, according to Schönfeld's observations, much turbidity, but not

the pronounced honey odour. In sweet wort they give a pleasant sourish odour and flavour. They are grouped together under the name *P. acedulefaciens*. Other varieties have been described by Schönfeld giving a red colour to lager beer. By inoculating a pure culture of a species producing strong turbidity into pasteurised beer which is allowed to stand, he showed that in most cases only a sedimentary growth developed, but if, on the contrary, carbon dioxide is passed through the beer, freely swimming bacteria develop which produce turbidity. This observation agrees with those made by Reichard in practice.

The fact that cases do occur in which lager beer contains bacteria appearing to possess the characters of true beer *Sarcina*, but having no recognisable influence either on the clearness, the odour, or flavour of the liquid, has since been confirmed in the author's laboratory. Experiments have proved that in isolated cases growths of one and the same species have caused diseases in one beer and not in another. From a number of individual observations of the associated conditions, the following conclusions may be drawn:—Species may be isolated from yeast and from lager beer capable of development, which appear to be incapable of exciting any disease whatsoever in the latter. The extent to which such organisms occur in beer is not yet known with certainty. Amongst the true disease species, a given organism appears to be unable to produce the special disease under all circumstances, even when the conditions are favourable for the reproduction of cells. According to our present experience, the most likely assumption appears to be that this is caused by the condition of the liquid at the time when the contamination with bacteria took place. According to the published experiments, the possibility cannot, therefore, be excluded that these organisms are capable of variation like other bacteria, and the question arises how far they are able to retain their newly-won properties.

No proof has yet been given that foreign species can be so completely acclimatised that they may act as disease bacteria in lager beer.

Both the typical *Sarcina* and the *Micrococci* (*Pediococci*) are widely distributed in nature, and may easily be recognised

by the use of the usual liquids and gelatines. Certain materials, for example, horse urine and dung, appear to be particularly rich in pronounced *Sarcina* species. Their presence can easily be verified also in malt and malt dust. It has not proved possible as yet to determine the natural haunts for the beer *Sarcinæ*. One reason for this is that such species cannot be distinguished from others that do not attack the liquor, in an ordinary micro-biological analysis. The only accepted conclusions are that all true beer *Sarcinæ* that have been exactly investigated cannot thrive in alkaline substrata (ammoniacal liquids or gelatine); that they form whitish masses in streak cultures, and on the surface of stab-cultures; that they belong to the facultative anaërobies; and further, that specially favourable conditions for their development are to be found in badly saccharified wort, and to some extent, according to Miskowsky (as in the case of many other bacteria), in malt extract with a high content of dissolved albuminoids (especially albumoses and peptones); in such a liquid they may remain for a long time unaltered; and, lastly, that, like many other bacteria, they appear to be checked by a large amount of hop constituents.

It follows that it is impossible to distinguish by any general test whether *Sarcina*-like bacteria in yeast or beer are able to produce a disease in beer. To answer this question we must proceed experimentally—a difficult investigation taking up much time. It would, however, be obviously foolish to neglect the usual test for *Sarcina*-like bacteria in yeast and beer, for if they are observed there is always a possibility that dangerous species may be present. It appears to be thoroughly established by experience that, in the early stages of fermentation, a weak growth of such bacteria may be concealed, and at present the problem is to provide means whereby the analyst may be able to detect minute quantities of these organisms. Amongst such means may be adduced Claussen's method for the treatment of yeast with minute quantities of acid ammonium fluoride which checks the yeast cells, so that a subsequent infection in wort-gelatine mainly gives a growth of *Sarcina* colonies. The liquid prepared by Bettges and Heller may also be used; it consists of sweet wort completely

fermented by the addition of yeast; starch is then added, and after clearing it is neutralised with ammonia, and reduced to an alcohol content of 4 per cent. The sample is mixed with this liquid, and the development observed in the sealed preparation. It will be found that of bacteria, only *Sarcinae* come to development. In the author's laboratory, for many years past, an addition of neutral yeast water (preserved in flasks along with an excess of calcium carbonate) is made to the sample taken at the end of the principal fermentation. After two days' standing the *Sarcinae* present have multiplied sufficiently to be easily recognisable under the microscope. Until investigations have gone far enough to enable us to prove whether the haunts of these disease germs lie outside the plant, efforts must be directed in practice to finding their haunts within the plant. We must bear in mind the limits of our present knowledge, and we must not forget that without pure culture experiments direct observation of *Sarcina*-like germs in the plant itself (for example in the vats) suggests a much greater probability that these growths are dangerous in practice, than if a large number of germs of similar microscopic appearance had been observed, with the help of plate-cultures, in the air surrounding the brewery or in the water used in the plant. By means of a properly organised system of disinfection, and often without the application of antiseptics, such growths may be entirely suppressed. It is an entirely false impression that the beer *Sarcinae* described in this section cannot be fully excluded, a mistake that has arisen because it has proved impossible to distinguish between many of the organisms of this large group of bacteria occurring in air and the true disease species of beer.

Investigations carried out in the author's laboratory on bright wines have frequently brought to light vigorous growths of *Sarcina* forms, and at the same time the wine has assumed a peculiar odour, which resembles to a remarkable extent the odour and taste of beers in which such growths occur.

#### 10. The Fermentation of Tobacco.

During the fermentation which dried tobacco leaves undergo, a number of organisms are present, and it is a natural

assumption that these play a part in producing the successive alterations in the leaf material. During fermentation the temperature gradually rises, and it is attempted in various ways to limit the temperature to about 50° C. The effect of fermentation is that aromatic bodies are produced in the leaves, and simultaneously part of the nicotine, according to Behrens, disappears. Suchsland was the first to investigate the micro-organisms present in fermenting tobacco. He attempted to improve its quality by introducing pure cultures of selected species of bacteria. Nothing further is known with regard to these species. More recently, Behrens, Vernhout, Koning, and others have described some of the vast number of species that are present, and Koning found by parallel experiments that an inoculation with certain pure cultures selected from fermenting tobacco, partly aërobies, but chiefly facultative anaërobies, exercised a favourable influence on the aroma and flavour of the tobacco. In the same way the after-fermentation, which takes place when the leaves are packed together, appears to be due to the action of micro-organisms. A contrary view has been expressed by O. Loew, who attaches no importance to micro-organisms in the fermentation, but seeks the active causes in the oxidising enzymes, which he proves to be present in the leaves. H. Jensen, as well as Splendore, found that leaves which had been heated in a current of steam (90°-100° C.) showed every sign of a good fermentation, and that this was not prevented by treatment of the leaves with mercuric chloride, formol, and chloroform, which certainly appears to confirm Loew's conclusion. Behrens bases upon his observations the belief that micro-organisms play a part in the fermentation, a view the correctness of which is rendered more probable by the results of Schloesing's experiments on the fermentation of snuff tobacco. The experiments undertaken in the author's laboratory with parallel fermentations carried out with both American and African tobacco lead to the conclusion, especially when facultative anaërobies are employed, that certain species do play a part in determining the aroma and flavour of tobacco.

### 11. Iron and Sulphur Bacteria : Nitrifying Bacteria.

The bacteria described in this section are of particular interest, because they possess the property of oxidising inorganic substances.

In microscopical examinations of water we often meet the characteristic forms of *Crenothrix Kiihniana* (Fig. 31), or spring pest, described by Cohn and Zopf.

This ferment occurs in all water containing organic matter, and sometimes it multiplies to such an extent that it may render the water unfit for use. Thus, according to Zopf, great calamities have been caused by this fungus in the water supplies of Berlin, Lille, and certain Russian towns. In consequence of its power of storing iron compounds within its walls, it forms red or brown flakes in water. Its forms are very beautiful ; it occurs in the form of motionless cocci or gonidia (*a-f*), which by division and formation of viscous matter form zoogloea (*g*) ; these cocci frequently grow to articulated filaments, which are provided with distinct sheaths (*h, i-r*) ; they then increase in thickness towards their free end, and when they reach a certain age, they divide within the sheath into smaller fractions, which become round and issue either as rods, macro- or micrococci.

*Leptothrix ochracea* is a widely distributed iron bacterium with colourless cylindrical cells connected in threads and surrounded by a sheath, which at first is thin and colourless, but afterwards, by accretion of hydrated oxide of iron, assumes a yellow or brown colour. Oval and motionless gonidia develop in the threads. The empty sheaths may form large yellowish-brown deposits in water containing iron.

*Cladothrix (Sphaerotilus) dichotoma* is also of frequent occurrence. Its cells are surrounded by a similar thin sheath. By displacement of single rods in a filament, false branching takes place. The rods are finally set free, and are then provided with cilia, with which they swim about until they settle down, and grow into new threads. These iron bacteria are commonly met with in water containing the soluble basic ferrous carbonate. According to Winogradsky this salt is oxidised by the bacteria and ferric oxide is deposited on the

sheath. The great deposits of iron ochre found in nature are probably to be partly accounted for by the activity of such bacteria. According to Molisch and others, they can also digest considerable quantities of manganese.

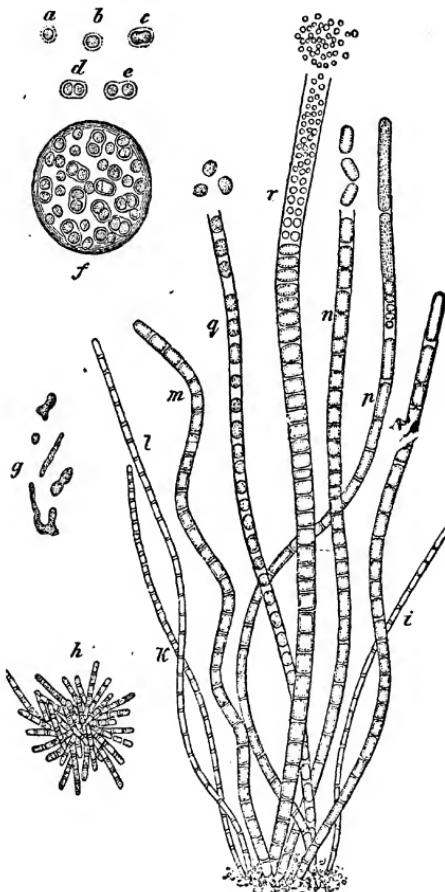


Fig. 31.—*Crenothrix Kühniana* (after Zopf).—*a-e* (600 : 1), Cocci in different stages of division; *f* (600 : 1), small, round cocci-zoogloea; *g* (natural size), zoogloea; *h* (600 : 1), colony of short filaments composed of rod-like cells, emanating from the germination of a small collection of cocci; *i-r*, filaments, partly straight, partly spirally curved (*i, m*), of very varying thickness, with more or less pronounced contrast between the base and apex, and different stages of division of their members and sheaths; the sheathed filament *r* shows short rods at the base, which higher up are divided into small cylindrical pieces; at the apex the cocci are seen arising from the longitudinal divisions of the cylindrical discs.

*Sulphur bacteria* occurring in water, many of which produce a red-colouring matter, have been described by Cohn, Warming, Engler, and especially Winogradsky. Under the microscope they are distinguished by the roundish bodies they contain, strongly refractive to light, and consisting of pure sulphur. They are aërobic, and occur especially in waters containing sulphuretted hydrogen. This substance is oxidised by the bacteria, and the sulphur split off is stored in the cells. Among the thread-like species the *Beggiatoa alba* may be mentioned. It occurs in cylindrical filaments without sheaths, which have a crawling motion, rotate round their longer axis, and swing from either end. They may expand to a great length. If the liquid contains sulphuretted hydrogen the filaments will be found to contain rounded grains of sulphur, strongly refractive. The threads divide by means of cross sections, and if no sulphuretted hydrogen is present they break up into single pieces, and gradually die off.

An important part is played in nature by bacteria which convert ammoniacal salts into nitrates; they are highly important for the nutrition of many plants. Schloesing and Müntz first described them; their observations were confirmed by Winogradsky, who made use of pure cultures. Among these nitrifying bacteria, as they are termed, there are some which oxidise ammonia into nitrous acid (nitrite bacteria), which is converted by other species into nitric acid (nitrate bacteria). As already mentioned, in an earlier section, these bacteria possess the power of living entirely without organic food, and in culture experiments in the laboratory they are grown in solutions of inorganic compounds. They form cocci and short rods, aërobic, motile, or motionless; they may grow in the dark, although they assimilate carbon dioxide from the air. Saltpetre may again be attacked by the denitrifying bacteria, which are capable of decomposing saltpetre in presence of organic substances with evolution of free nitrogen. The nitrifying bacteria also cause the efflorescence of nitre from walls, which often brings about the decay of brickwork, snow-like masses of calcium nitrate becoming detached. This evil can be remedied by means of antiseptics.

## CHAPTER IV.

## MOULDS.

CERTAIN moulds as well as bacteria are of industrial importance, and have been applied in the form of properly selected pure cultures. On the other hand, a large number of species are known that produce objectionable diseases in the different branches of the fermentation industry. They select as their habitat the vessels, tools, rooms, the green malt, and the quiescent masses of yeast, especially top-fermentation yeast. As shown below, the moulds usually play a subordinate, but nevertheless a quite important part. A close examination of mould growths taken from the ceiling or walls of a fermenting room, or from the edge of a vessel, soon shows that they scarcely ever consist of an unmixed growth of moulds. Amongst the mycelia, bacteria and yeast-like cells can almost always be found. The hyphae of the mould plant project outwards, carrying foreign germs with them, and these, in their exposed situation, are easily swept away either by air currents or by workmen. All kinds of micro-organisms occur on the raw material during the malting process. If moulds are usually considered to be the worst enemies, it is due to the fact that they are visible to the naked eye, and so attract special attention. If we judge by numbers, then bacteria must certainly take the first place, for they are always present in great numbers on green malt. It may even be considered doubtful whether the greatest influence on the product must be attributed to the moulds (*Penicillium*, *Aspergillus*, etc.), when these are met with in a state of vigorous development on malt, or whether it is not far more probable that the numerous organisms accompanying them play the most important part. It is doubtful whether the so-called "mouldy" smell of beer is caused by moulds. Observations

made in the author's laboratory point rather to the action of bacteria. In distilleries and yeast factories, on the other hand, moulds have been known to appear even during fermentation. Growths of *Oidium*, *Chalara*, *Dematiuim*, etc., are, for example, found on the surface of the yeast layer in the vat, and the yeast-like cells which are produced by these fungi—and by certain species of *Mycoderma*—which bear a striking resemblance to true yeast cells—can frequently be observed multiplying in the upper yeast layer. They may be skimmed off along with the yeast, and thus the author has often found a fine white deposit on the surface of pressed yeast, which most frequently consists of a mould mycelium, belonging principally to forms resembling *Oidium*, *Chalara*, and *Dematiuim*. It is quite possible that when these plants form a dense layer on the surface of the yeast-mass, they retain by respiration a portion of the free oxygen which is necessary to enable the quiescent yeast to remain alive. Even here, without exception, bacterial growths were observed.

Judging from practical observations, a growth of mould nearly always serves to indicate that other organisms of a more injurious and more active character are developing. It is, therefore, of great importance that the walls of the fermenting rooms should be smooth; this is effected with the greatest certainty by employing the enamel paint now so much in use.

The moulds are also of direct significance to the dairy industry. The experience of recent years has shown that amongst the great diversity of forms which make their appearance in this industry, some of which are of the nature of unbidden guests, many could contribute in no small measure to the improvement of the quality of cheese if proper means were adopted. Amongst the useful forms may be mentioned *Aspergillus oryzae* from Japan, which, on account of its powerful diastatic enzyme, is used for the manufacture of sugar from starch; *Amylomyces*, introduced from Indochina, and employed in European distilleries; and *Citromyces*, similar in appearance to *Penicillium*, used in the preparation of citric acid from grape-sugar.

The moulds, many of which represent stages in the life

history of higher fungi, occur in the form of vigorous growths easily visible to the naked eye. Each species has a very characteristic appearance, which is due, not only to its structure, but also to the colour which it affects at a particular stage of its development, varying in shade from the purest white to the deepest shades of colour.

The individual cells of which the body of the mould plant is built up consist of a cell-wall or membrane, together with the cell-contents, which consist essentially of protoplasm, vacuoles, and various contents, of which the most important is the cell-nucleus. The membrane is composed of a substance known as fungus-cellulose, differing, as a rule, in its chemical reaction from the cellulose of higher plants. Both inner and outer surfaces are subject to gradual thickening, and with increasing age become impregnated with deposits of colouring matter, and incrusted with crystals, especially of calcium oxalate.

The protoplasm (or Cytoplasm) consists of a homogeneous viscid substance packed with minute granules. This constituent of the cell determines the growth, and a part of it forms a thin layer lining the inner surface of the cell-wall. The protoplasm in living cells is in constant motion; in certain cases (e.g., the young sporangiophores of species of *Mucor*), the motion is sufficiently active to permit of its observation through the microscope. In very young cells the protoplasm occupies the entire cell space. Later on vacuoles appear, and at the same time different kinds of corpuscles, amongst which the "crystalloids" may be cited, consisting of albuminoid substances, which may perhaps be regarded as products of secretion. There are also the widely distributed fatty oils and fats, which are especially abundant in the reproductive organs and the resting cells. A very important part of the protoplasmic contents is the nucleus, a small rounded body, which, by the addition of a suitable staining fluid, becomes very prominent, and under the microscope can frequently be seen to include an inner and more highly coloured portion, which is called a nucleolus. By using special staining methods, it has been shown that the nuclei are capable both of division and of fusion, processes which are directly connected with the different stages of development which the

vegetative and reproductive organs of the fungus pass through. We shall return to this subject when we come to a description of yeasts. The cells form the mycelium of the fungus. This is composed of branched or unbranched threads (Hyphae), usually provided with transverse walls, *the growing region* being always at the apex. Outgrowths may arise from the older cells which develop to form lateral branches. Transverse cell-walls are usually absent in the mycelium of fungi belonging to the *Mucor* spores. Thus, the whole of the vegetative portion of the mycelium of these fungi, with its intricate network of branches, consists of one single cell. Amongst the members of this genus a second form is known. Under special conditions, the submerged portion of the mycelium divides into separate cells, which break away from each other, become rounded off, and then give rise to protuberances which themselves repeat a similar mode of growth. This is the so-called *spherical yeast*, which grows in the same way as true yeast. The same modification has been found to occur amongst many other forms of moulds, and there exist, moreover, forms exhibiting every intermediate stage down to those in which the mycelium is almost entirely suppressed, and the greater part of the growth takes the form of budding.

Many moulds form peculiar resting organs, in which the walls are thickened and the mycelia closely packed together, surrounded by a dark, sometimes felted, pseudo-cortex formed by the outer hyphae. In this way the small hard bodies, known as *Sclerotia*, are formed. These are packed with stores of reserve food material, and may retain their vitality throughout a lengthened period, thus serving to ensure the maintenance of the species during conditions which are unfavourable to growth. Passing on to the manner of reproduction of the moulds, we are brought face to face with the remarkable fact that these organisms, although occupying so low a position in the vegetable kingdom, possess in many cases, not one only, but several entirely different methods of reproduction. Every stage of complexity may be met with, from the simplest forms of reproductive organs to the most highly developed. In *Oidium lactis* reproduction is effected without the aid of any specialised organs. The threads of the mycelium divide quite simply

by means of transverse walls into short cylindrical pieces, so that finally the whole plant is transformed into numbers of "Oidia," each one of which is capable of giving rise to a new individual.

The reproductive bodies are termed **spores**. Of these, the simplest kind are the **conidia**, which are formed by constriction from one or more of the mycelial hyphæ, termed the conidiophores. This constriction may take place in two ways. In the first case, under the conidium first formed at the end of the thread a new growth develops, the new piece swells out and forms a new conidium, and so the development continues, the successive spores being produced basipetally—*i.e.*, towards the base of the thread. This is the method of spore-formation in *Penicillium* (see Fig. 33). In the second case, the spore first formed at the end of the thread expands at its upper end, and is constricted to form a new conidium, and the development proceeds in this way from the base of the thread upwards (basifugal). These conidia may, however, also develop spores sideways. This development is similar to the budding of yeast cells. It has been observed in *Cladosporium*. The conidiophore may be branched, or may assume a still more complex structure. Reference to Fig. 33, for instance, will show the condition in *Penicillium*, where the numerous and minute conidia are arranged in long chains, each at the tip of an individual branch. An altogether different kind of spore-formation is that which results in the production of **zygospores**, such as are met with in *Mucor*, and described in detail further on. The remarkable feature of this kind of spore-formation is that it is brought about by the fusing together of two cells, between which there would appear to exist a definite distinction of sex. In contrast to these, we find other spores produced in the interior of certain cells. Here, again, *Mucor* serves us with an example (Fig. 35), where each mycelium carries a **sporangium** in the interior of which a large number of oval spores are formed round a column (columella), which are liberated by the rupture of the wall.

Quite a different class of internal spore is met with in such forms as *Aspergillus* (Fig. 34). Here a small and definite number of spores are produced in a tubular cell (**ascus**). In

many fungi a large number of these spore tubes may occur combined to form fruit-like clusters, as seen in Fig. 34.

Lastly, it may be stated that there are yet other forms of reproduction occurring in the moulds. We must pass over their detailed description, and only mention that many species possess specially constituted resistant spores (to which class the zygosores belong), which enable the fungus to preserve its vitality for a long period even under the most unfavourable conditions. Although apparently very fragile, in reality they share with bacteria the power of offering a very stubborn resistance to attacks from without, and can maintain their vitality for many years.

When ripe, the spore is capable of germinating. An example of the development of a *Penicillium* conidium is given in Fig. 33. The very minute spherical cell first absorbs water, and its volume soon increases to many times the original size. The cell-wall then commences to bulge out at a certain point, the outgrowth gradually lengthening into a thread-like process, which is soon cut off from the mother cell by a transverse wall. The growth of the filament continues, branches are given off, and new transverse walls are formed. Where the spore is furnished with a strongly thickened outer coat, as in the zygosore of *Mucor* (Fig. 35), it is ruptured by the pressure exerted by the underlying inner coat which grows out to form the germinal hypha.

The spores of fungi are more resistant than the mycelium. E. C. Hansen has shown, for instance, that the spores of a species of *Penicillium* were capable of germination after having been kept dry for twenty-one years. According to the same author, a crop of *Aspergillus glaucus* was obtained from dried spores after the lapse of sixteen years. The spores of many species can endure very high temperatures, especially when in the dry state. Thus dry *Penicillium* spores withstood a temperature of 120° C., but when damp they were killed at 100° C. The remarkable power of resistance to high temperatures, which range far beyond that at which albumen coagulates, has been explained by assuming that the albumen present in the spores is so highly concentrated that the small quantity of water present is insufficient to bring about coagu-

lation. The spores also show an extraordinary power of resistance to low temperatures.

The moulds differ in a marked degree in their sensibility to poisons. *Botrytis*, for instance, is killed in a solution of formaldehyde four times weaker than that which destroys *Penicillium*, a fungus possessing extraordinary power of resisting many poisons. Thus, it will withstand a solution of mercuric chloride sixteen times stronger than that which will kill *Botrytis*. *Botrytis*, on the other hand, can withstand a solution of a poisonous silver salt four times as strong at that which kills *Penicillium*.

The mould species described in the following pages have been selected partly on account of their growth forms (representing types of development), and partly because of their occurrence in the fermentation industry; they belong to different sections of the system of "higher fungi."

The higher fungi are grouped together as Oomycetes (to which belongs the fungus causing the potato-disease, *Peronospora* or *Phytophthora*), Zygomycetes (to which *Mucor* belongs), Ascomycetes (including *Penicillium*), and Basidiomycetes (to which groups the mushrooms belong). There are in addition a large number of fungi which are only known to us in their conidial form, such as *Oidium*, *Dematioides*, *Monilia*, and *Cladosporium*, which, it is assumed, represent a stage in the life history of some of the higher fungi, as has indeed been definitely proved in certain cases. These are known as "*fungi imperfecti*," a title which is not intended to be a reflection on the fungi, but solely to define our lack of knowledge respecting them.

The general features of the nutritive physiology of fungi will now be dealt with. The several forms make very varied demands on the chemical constitution of the substratum. Some are able to absorb certain of the chemical elements as such, but the majority assimilate them in the form of organic or inorganic compounds. A large number of fungi are saprophytic, living on decaying substances; others adopt a parasitic mode of life, and derive their nourishment from the bodies of living organisms. In dealing with nutrition, we distinguish between the process of assimilation, the conversion of the absorbed food substance into body substance, and that of disassimilation, the

different phenomena of decomposition and splitting up due to the vital activity of the organism. The most important of the vital functions in this respect is that of respiration. The two processes are *intimately related to each other*. During respiration a large number of different nutrient substances are transformed, and, in common with all other forms of life, the final products are carbon dioxide and water. Amongst the products formed under conditions of incomplete oxidation, mention may be made of oxalic acid, which is of remarkably wide occurrence in fungi. In addition to the usual respiration of oxygen, a further form of oxidation in the absence of oxygen may take place, to which the term intramolecular respiration or respiration by dissociation has been given.

The following facts may be of interest in regard to the individual substances that play a part in the nutrition of fungi:—*Potassium* appears to be essential to the growth of moulds, particularly to the formation of their reproductive organs. Within certain limits this element forms a useful food for yeast, and the fact must not be overlooked that it also plays a part in the nutrition of bacteria. *Magnesium*, amongst the alkaline earths, is necessary for the generality of moulds, as well as for yeasts and many bacteria. The formation of colouring matter in many bacteria has been shown to depend on the presence of magnesium in the nutrient fluid. It may further be assumed that many fungi require *Calcium* in order to attain perfect development. *Iron* appears to be required by moulds either as a food or as a stimulant, and, especially in the form of the sulphate, it exerts a favourable influence on the propagation of yeasts. We can assume that *Sulphur* is an essential element, since all albuminous substances contain sulphur; it is usually added to the nutrient solutions in the form of a sulphate. Many fungi, however, seem to be capable of developing in nutrient solutions to which no addition of sulphur has been made. Sulphur plays a very important part in the metabolism of the sulphur bacteria. *Phosphorus* is assimilated by fungi in a variety of compounds, and is, without doubt, a necessary constituent of their food. The fluorescence exhibited by many bacteria depends upon its presence. *Nitrogen* is, as is well known, of the greatest

importance as a foodstuff for fungi, and is assimilated by them in very varied forms. Some fungi are capable of absorbing and fixing nitrogen in its free state; others obtain *their nitrogen in the form of inorganic compounds* (ammonium salts, nitrites, nitrates), including species which can only absorb nitrogen from inorganic substances. The most important group of fungi, however, are dependent on organic compounds containing nitrogen, such as amides, peptones, albumoses, etc. The first two groups must obviously have access to a supply of carbon, and this is often the case with the third group, for in many cases the nitrogen bound up in the organic substance can only attain its full value as a food in conjunction with other sources of carbon. Both elements, however, usually occur together in the same chemical substance, and it is moreover impossible to draw a sharp distinction between any of the above-mentioned groups. In regard to the relative food value of the substances themselves, it may be noted that, on the whole, the ammonium salts constitute a better source of nitrogen than the nitrates. The amides also play an important part in the nutrition of many fungi.

The greatest diversity exists as to the sources from which the fungi may obtain their *carbon*. Sugar, tartaric, acetic, oxalic, and carbonic acids are amongst them. In these, as in other cases, the value of the nutritive matter varies according to the other conditions of nourishment. The amount of aëration is an important consideration, since oxygen facilitates the absorption of certain substances, and is stimulating to some species, but acts restrictively on others. Temperature, like aëration, may react in either direction. The nature of the nitrogenous compound, too, has a definite bearing on the availability of the source of carbon. In the case of yeasts, acetic acid forms an excellent carbon food for the *Mycodermae*. Citric and tartaric acids form specially good food for certain *saccharomycetes*, and, again, malić acid can be assimilated in considerable quantity by certain species. Many bacteria also take up organic acids in the presence of nutritive salts. Glycerine and mannite are good sources of carbon for the moulds. It is, however, the **carbohydrates** which, as is

well known, form the principal and most important food-stuffs. We shall return to these in the subsequent chapter on yeasts.

Speaking generally, one may assert that constructive activity predominates during the nutrition of green plants, whilst destructive activity predominates in the case of moulds ; the enzymes which are of such general occurrence are the special destructive factors.

It may be remarked that certain substances, which are not necessarily incorporated by the fungi, are nevertheless of importance to them. The influence which these exert on the metabolism and growth of fungi suggests the term "chemical stimuli." The presence of water and oxygen, for instance, are essential in bringing about the germination of spores, but it frequently happens that germination can only take place if certain substances are also present in solution. Klebs has shown that the spores of *Aspergillus repens* will not germinate either in pure water or in inorganic nutrient solutions, or even on peptone, unless some inorganic salt, such as saltpetre, is added, but that, in a 0.5 per cent. solution of grape-sugar, germination does take place. Light also acts as a stimulant in certain cases, whilst in others it may have a retarding effect. It has been ascertained that minute doses of certain poisons have the effect of stimulating the growth of fungi, and of accelerating fermentative phenomena, an action which may, perhaps, be ascribed to physiological reaction on the part of the organisms concerned. Thus, a small quantity of zinc sulphate (0.002 per cent.) added to a solution of sugar and inorganic salts has the effect of making the growth of *Aspergillus niger* twice as strong as under ordinary conditions. Copper sulphate has a corresponding action under certain conditions. In the case of yeasts, the addition of the merest trace of substances such as mercuric chloride (1 : 500,000), iodine, potassium iodide, and chromic or salicylic acids has a very beneficial action on the fermentation. Lactic acid bacteria, grown in milk free from casein, are also stimulated by the addition of minute quantities of mercuric chloride.

The interesting observation has recently been made that products formed by the metabolism of the fungi have a stimu-

lating action on their growth, provided that certain conditions are observed, and the supply of nutrient substances is maintained.

These observations naturally lead to a consideration of the question of **external influences**, and their action on fungi in general.

An essential condition of growth of all organisms is the presence of **water** in such quantity as to create and maintain in the cells a condition of turgescence—*i.e.*, a hydrostatic pressure within the cell which keeps the protoplasmic lining in direct contact with the cell-wall. They require, however, only a minute quantity of water during the resting stage—*i.e.*, in the state of spores—and frequently these organisms can withstand complete dehydration. It has already been mentioned that yeasts can be preserved in a dry state for a very long time, and spores of some of the moulds for several years. The concentration of the food, which is dependent on the amount of water present, plays an important part in the development of individual species. It is well known that temperature is also an important factor.

Growth is only possible above a certain minimum temperature, and it is accelerated with a rising **temperature** until at a certain optimum the organism attains its greatest activity. From this point on, if the temperature is still allowed to rise, the growth becomes less and less pronounced, and, finally, at a certain maximum temperature, ceases altogether. Different species exhibit very varied behaviour in regard to these three critical points, as is shown by the following examples:—

A number of yeast species have their minimum at 0·6° C., optimum at 28°-30° C., and maximum at 34°-40° C., whilst the corresponding figures for—

	Minimum.	Optimum.	Maximum.
<i>Penicillium glaucum</i> are	1·5° C.	25° to 27° C.	31° to 36° C.
<i>Bac. subtilis</i> ,	6° C.	approx. 30° C.	50° C.
Acetic bacteria,	8° C.	18° to 33° C.	30° to 36° C.

The limits of temperature may, moreover, vary in regard to the different organs of one and the same species (instances are found amongst the yeasts), and finally the results obtained

in connection with any one particular species will vary according to the nature of the food supply.

In general, it may be remarked that organisms can withstand temperatures below the minimum without permanent injury. Thus, according to experiments by Schumacher, yeast cooled down to  $-113^{\circ}$  C. was not killed. Macfadyen subjected bacteria to gradual cooling down to  $-172^{\circ}$ , and even to  $-190^{\circ}$  C., for a period of twenty hours, and mould spores to  $-210^{\circ}$  C. without killing them. On the other hand, a slight increase in temperature above the maximum is often fatal. It is only spores of bacteria, particularly when dry, that are resistant to high temperatures. Many spores may, under these conditions, be heated for a short time to  $140^{\circ}$  C. without injury.

It is an interesting fact that some species of bacteria capable of thriving at a temperature of  $0^{\circ}$  C. exist, and others, as stated, develop best at  $50^{\circ}$  C., or even  $70^{\circ}$  C.

Light is of vital importance for the fungi, although not to the same degree as is the case with green plants. The injurious effect which light has on the growth of bacteria, for instance, is now widely recognised, and it has been ascertained that for fungi in general both the nature of the nutritive medium and the temperature have a regulating effect on the action of light. From the physiological standpoint, the most active rays of light are the blue, violet, and ultra-violet rays.

The natural purification of rivers is generally accepted to be due to the germicidal effect of light.

Kny found that subdued light has no influence on yeast. Lehmann, who used the intensive light of an arc lamp, but experimented at low temperatures, arrived at the same result. He found that light had a retarding influence on the multiplication of yeast cells at  $18^{\circ}$  C. and above, and that these were killed by prolonged exposure to direct sunlight, whilst diffused daylight delayed the process of budding.

Many moulds can endure sunlight without injury, but intense illumination frequently restricts the longitudinal growth of the mycelial threads. Some species produce only mycelia in the dark, the reproductive parts requiring light for their development. In the case of *Botrytis cinerea*, the

formation of conidia is retarded by the more refractive blue and violet rays.

A number of experiments have been undertaken to test the effect of **electricity** on fermentation organisms. Results have shown that the electric current has no influence, if care is taken to prevent the heat developed by the current from influencing the bacteria. Whatever significance the electric current may be said to possess, such, for instance, as the preservation of fermented liquids, must be ascribed to the chemical effect of the current. So far as its action in regard to wine is concerned, the evidence is contradictory. By the treatment of water the number of germinable spores has been considerably reduced. The action of **ozone** on water has already been referred to. In the treatment of yeast in a fermenting fluid, it has also been shown that the electro-chemical changes brought about by the current have a fatal effect on the yeast.

It is a well-known fact that fungi are highly resistant to the influence of **pressure**. Bacteria, for instance, are found in the ocean at such a depth that they must be exposed to a pressure exceeding 100 atmospheres. According to Melsens, yeast cells can withstand a pressure of 8,000 atmospheres.

Chlopin and Tamman also found that bacteria, as well as yeast and moulds, could endure a pressure of as much as 3,000 kilos. per square centimetre. A rapid rise of pressure to this point, followed by instantaneous release produced only a slightly injurious effect. The fermentative activity was only retarded under long-continued pressure. An attempt has been made to utilise the combined action of pressure, and oxygen or carbon dioxide to sterilise bacterial liquids, without, however, much success.

With regard to the influence of **rest** and of **motion**, it may be observed that the organisms of fermentation thrive well both in and upon quiescent substrata. Yeast also, as is well known, not only withstands active motion in its culture medium, but responds to it by a more active development. The different species of bacteria respond in varying degrees to violent shaking. Thus cholera bacilli cannot withstand shaking, but no effect is produced on typhus bacilli. Buchner

and Rapp have shown that, whilst a slight motion has a beneficial effect on the fermentative activity of yeast, violent mechanical shaking has the effect of materially reducing this activity, the reduction being the more pronounced the poorer the fermenting medium.

The action of antiseptics on fungi is intimately connected with the activity of the organism and with the temperature. High temperatures augment the injurious effect of the reagent. The degree of concentration of the substance is also of importance, for, as already mentioned, minute quantities exercise a stimulating effect. In somewhat greater concentration the growth is retarded, whilst considerable quantities are fatal.

In apparent contradiction with the above stands the remarkable fact that more concentrated solutions of antiseptics sometimes have a less effect on bacteria than weak solutions ; thus, a 3.5 per cent. solution of cupric chloride killed the spores of the anthrax bacillus in a shorter time than that required by a solution of four times the strength.

It is also known that different species exhibit a very varied resistance to the same substance, and to the same quantity of that substance. Substances, moreover, which form suitable foods for some species are poisonous to others. A great diversity of behaviour in regard to a particular poison is shown by one and the same fungus in its various stages of development ; bacteria are more easily killed in the vegetative than in the spore condition. Some fungi can withstand a certain amount of poison without their growth being restricted, but under such conditions, or even in the presence of a smaller quantity of antiseptic, they are unable to form reproductive organs. The fungi possess the interesting power of adapting themselves to poisons when they are treated to gradually increasing quantities. An example has been mentioned in Chap. i. (hydrofluoric acid).

The **chemical constituents** of fungi are of a most varied nature. Water is the only one occurring almost always in relatively large quantities, the amount in bacteria representing about 80 per cent. ("Mother of Vinegar" forms an exception, and contains 98 per cent.) The yeast group contains from

40 to 80 per cent. The moulds and the vegetative parts of higher fungi contain from 80 to 90 per cent., whilst in the resting and reproductive organs the percentage is much lower.

In common with all organised bodies, the fundamental chemical constituents of fungi are the elements carbon, hydrogen, oxygen, and nitrogen. The relative amount of ash is found to vary enormously; the mycelia of *Aspergillus* and *Penicillium* contain about 6 per cent. of ash. The mineral substances which may be regarded as essential constituents of fungi are sulphur, phosphorus, chlorine, potassium, calcium, magnesium, iron, manganese, and sodium. Sulphur and phosphorus are of special importance, as they form necessary components of the albuminoids. Some idea of the importance of the phosphorus present may be gathered from the fact that its proportion in the ash may often amount to half the total quantity. Chlorine, which apparently plays an important part in the nutrition of fungi, and which may be concerned in bringing about the dissociation of the nutrient fluids, probably occurs in all fungi.

Amongst the metals, potassium occurs in the largest proportion, whilst calcium and magnesium are present in smaller amounts. The same is true of iron and manganese, which, although occurring in comparatively small proportions, are nevertheless of the highest importance to the organism in the different phases of its life history.

Cellulose, the carbohydrate which is of such general occurrence amongst the higher plants, is found also in the walls of fungus cells, but the mucilaginous or viscous substances which often characterise the cell-walls of the former occur comparatively seldom. Yeast mucilage is, however, a well-known example of this. Foremost among the substances forming the contents of fungus cells must be mentioned the proteins or albuminoids, which are in all probability the actual carriers of the vital phenomena. They possess in addition a practical significance; yeast, for example, which is very rich in albuminoids may be used for the preparation of nutritive media to take the place of meat-broth, etc.

The nucleins may be regarded as constituting a special

class of albuminoids. They have a most important *biological* significance, owing to the fact that the nuclei, as micro-chemical investigations have shown, are mostly composed of nuclein substance. In the case of yeast in which the reactions have been more closely studied, it was found that by treating them with pepsin the albuminoids in the cell were dissolved, but the nuclein compounds remained unchanged.

Our knowledge in regard to the other albuminoids occurring in the cells of fungi is confined at present mainly to the investigations which have been made on yeast.

Closely associated with the albuminoids, and, indeed, derived from them, are the **enzymes**, which doubtless occur in all forms of life. They may be separated from the living cell, and can still produce their characteristic effects in aqueous solutions. Each enzyme has the property of bringing about a definite and particular form of chemical change, and the process is further characterised by the fact that a small amount of enzyme is able to decompose a relatively large quantity of organic substance.

The result of the changes brought about by the action of the enzyme can be recorded in the form of a simple equation. According to H. Fischer, the enzymes may be classified as follows :—

I. Dissociating or splitting enzymes, by means of which a complex substance is split into its component parts. The action may be regarded as a form of hydrolysis, and is accompanied by the absorption of water. The biological significance of this enzyme consists in enabling the organism to convert substances which would otherwise be useless into simpler and soluble substances capable of diffusion.

The group includes :—(1) Carbohydrate-splitting enzymes, such as invertase, which converts saccharose into equal molecules of dextrose and lævulose ; maltase, converting maltose into two molecules of dextrose ; and lactase, which converts milk sugar (lactose) into equal molecules of dextrose and galactose. The sugars—saccharose, etc.—can only be converted by fermentation into alcohol and carbonic acid after undergoing inversion. The melibiases convert melibiose (a product of hydrolysis from raffinose) into one molecule of galactose and

one of dextrose. This enzyme is found in a large number of yeasts and moulds, and also in a few bacteria. The diastases also belong to this group. They convert starch into dextrin and maltose, and occur in fungi, such as *Mucor* and *Aspergillus*. Cytase acts on cellulose, etc.

(2) The glucoside-splitting enzymes, to which group emulsin belongs, an enzyme discovered by Liebig and Wöhler in 1837. This enzyme changes amygdalin, a glucoside occurring in bitter almonds, into benzaldehyde, hydrocyanic acid, and glucose.

(3) The fat-splitting enzymes (lipases), which decompose fats into glycerine and fatty acids.

(4) The albumen-splitting or proteolytic enzymes (proteases), including pepsin, occurring in gastric juice, which converts albuminoids into albumoses and peptone; trypsin in the intestinal secretions, which brings about further changes, and, more in our domain, the endotryptase of yeast.

II. The enzymes in this class have quite a different action from the foregoing. They include the oxydases, the effect of which is to split up molecular oxygen, and thus render it active.

Buchner and Meisenheimer have shown that oxydase occurs in acetic acid bacteria, and also in yeast.

III. Reducing-enzymes (catalases). These appear to occur in yeast and in certain bacteria.

IV. A special group, the fermentation enzymes, or zymases, the chemical action of which is entirely different. They bring about true fermentation, which has been defined by H. Fischer as an intro-molecular readjustment of oxygen, accompanied both by oxidation and reduction of the several carbon atoms, and an increase in the compounds of carbon and oxygen, the usual result of the process being the breaking up of a single molecule into several. In addition to the true zymase (or alcoholase), this group includes the enzyme of lactic acid fermentation. A further description of the former will be found in the chapter on alcoholic yeasts.

The activity of enzymes is influenced to a very considerable degree by temperature. The optimum for zymase lies at about 30°, for pepsin about 40°, and for the proteolytic enzymes (e.g., barley malt) at about 60° C.

The action of dilute acids and alkalies is also very varied. Thus, minute quantities of free hydrochloric acid ( $\frac{1}{3000}$  of normal acid) greatly increase the activity of invertase.

The action of enzymes is to a great extent analogous to that of hot acids (hydrolysis).

The formation of the enzyme by the fungus is dependent to some extent on nutrition, as already stated in the chapter on bacteria. *Aspergillus glaucus*, for instance, as Duclaux has shown, when cultivated on a solution of calcium lactate and nutrient salts, secretes diastase, but no invertase. On the other hand, on a solution of cane sugar and nutrient salts, invertase is produced, but no diastase nor any other enzyme that it is capable of producing. Only by cultivating the fungus on milk is it capable of producing clotting enzymes and casease. Similarly, it was discovered by Went that a species of *Monilia*, which possesses a number of enzymes, could only form certain of them, such as trypsin and the clotting enzyme, when substances capable of being split up were present. Fermi proved that several kinds of bacteria, when cultivated on media free from albumen, formed no albumen-splitting enzymes, and that *Bacillus subtilis* only produced diastase when fed with some form of peptone.

In fungi, moreover, a number of poisonous substances occur—ptomaines, toxins, etc. These are not to be regarded as produced exclusively by the higher fungi (toadstools, etc.), but occur also in the lower forms, such as rusts and smuts, which have brought about cases of poisoning. Reference may also be made to the extremely poisonous nature of *Aspergillus fumigatus* and *A. flavescens*.

The carbohydrates which have been shown to occur in fungi include glucose and laevulose; mannite is very widely distributed, and glycogen, to which we shall return in the chapter on alcohol-producing yeasts, generally occurs as reserve substance.

Fats and free fatty acids are found as reserve substances, and as secretions in many fungi. *Penicillium* and *Aspergillus*, for example, have been shown to contain from 4 to 5 per cent. of these substances. Yeast stores up fat along with glycogen, the former constituting from 2 to 5 per cent. of

its dry weight. Amongst organic acids, oxalic acid is widely distributed.

A whole series of colouring matters are found in the cells of these plants. The colouring matter present in so many of the bacteria is specially interesting, and has been shown to play an important part in their vital economy. The fatty colouring matters or dye-stuffs combined with fatty acids (lipochromes) are of frequent occurrence. Allusion may also be made to tannins (found in alcohol-producing yeast), resins, and ethereal oils.

### 1. *Botrytis cinerea* (*Sclerotinia Fuckeliana*) (Fig. 32).

*Botrytis cinerea* forms small greyish-yellow patches on moist decaying vegetable matter, and may also occur on wort. From the greyish-brown mycelium the conidophores are thrown up; these are perpendicular articulated filaments, generally arranged in tufts. They grow up to a height of 1 mm., after which the apical cell throws out near its point, and almost at right angles, from two to six small branches ( $C''$ ). The lower branches are the longest; these again give rise, at a short distance behind their apices, to one or more short side branches. The topmost branches are almost as wide as they are long. Thus a system of branches is formed shaped like a cluster of blossom or bunch of grapes. When longitudinal growth is at an end, the interior of the branches is separated from the main stem by the formation of a transverse wall close to the latter. At the same time the ends of the branches and of the main stem swell, and on the upper half of each swelling several small papillæ appear close together; these quickly increase to oval blisters, filled with protoplasm, and grow narrow and stalk-like at their base. When these conidia ( $C'$ ) are completely developed, the walls of the branches carrying them shrivel up, and the conidia are consequently brought so closely together that they form a loose, irregular aggregation, which readily falls off. If these clusters are placed in water, the conidia detach themselves from their stalks, and the envelopes of the branches, devoid of protoplasm, shrivel up or are only to be

found in traces ; their former place of attachment to the main filament appears only as a slightly raised scar. The member immediately below can now displace the shrivelled apex, grow upwards, and form a new cluster ; this may be repeated several times, whereby the conidiophores attain a

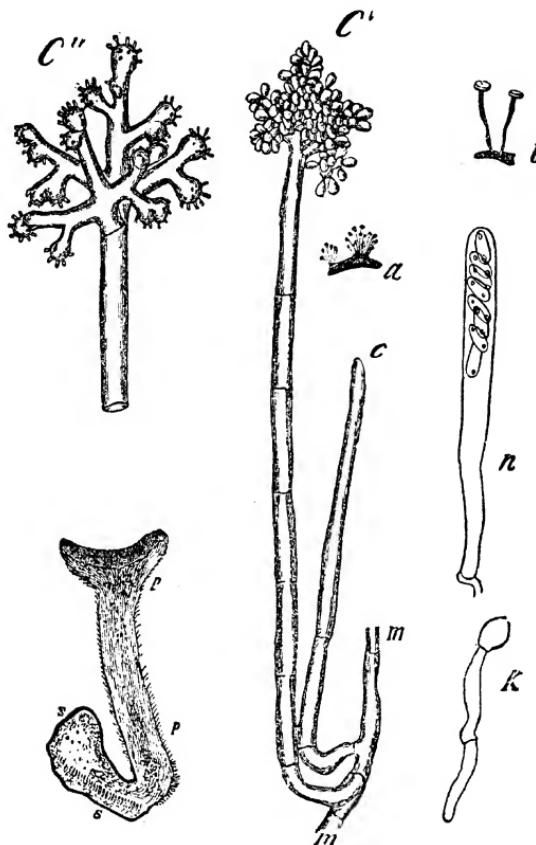


Fig. 32.—*Botrytis cinerea* (after de Bary).—a, b (natural size), Sclerotia, from which at a the conidiophores, at b the apothecia (fruits with ascii), are thrown out ; c, C, conidiophores (C', with conidia just ripe), springing from the mycelium filament m ; C'', end of a conidiophore with the earliest formation of conidia from the ends of the branches ; k, germinating conidium ( $\times 300$ ) ; p, s (slightly magnified), section through a sclerotium s, from which a very small apothecium ( $p, p$ ) is thrown up ; n, single ascus, with eight ripe spores ( $\times 300$ ).

considerable length. According to the observations of Rindfleisch, the formation of conidia takes place only during the

night. Klein and Lindner found that in daylight the more strongly refracted portion of the spectrum, the blue-violet rays, hindered the formation of conidia, whilst it is encouraged by the red-yellow rays. Under the constant influence of red-yellow light, and in total darkness, the production of conidia goes on both day and night. Under certain nutritive conditions the conidia and ascospores develop short threads, from which small, bright, round conidia are separated, either directly or on bottle-shaped basidia. These conidia are not capable of germination. If the mycelium has been cultivated for some time on a solid substratum which it is incapable of penetrating, short branches are formed, which by further and repeated branching have the appearance of closely arranged tufts or tasseled knots. They lie in close contact with the substratum, and form the characteristic organs of attachment.

Under certain conditions this mould can assume a peculiar dormant state, the **sclerotium** (*skleros* = hard) (see Fig. 32, *a*, *b*, *ss*). The hyphal threads branch out freely, and the branches intertwine themselves into a compact body of varying shape, circular or fusiform, and of varying size; the extreme ends of the filaments are brown or black, and the ripe, solid sclerotium thus consists of an outer black rind and an inner colourless tissue. These bodies, which were described by de Bary under the name of *Sclerotinia Fuckeliana*, occur as small black bodies on the herbaceous parts of many plants, where they live as parasites or saprophytæ. They are capable, after a long period of rest—lasting at least a year—of forming a new growth. If the sclerotium is brought into a moist place soon after it comes to maturity, the inner colourless branches break through the black outer rind and develop into conidiphores (*a*). If, however, the sclerotium is only brought into a moist place after it has been at rest for some time, a large tuft of filaments develops from the inner tissue, and these shoot up perpendicularly, and finally spread out to a flat, plate-shaped disc (*b* and *p s*); the ends of the filaments are arranged in parallel rows on the free upper surface of the disc; some of them remain thin, others swell up to club-shaped asci, and each of these asci forms in its interior eight oval spores (*n*). The mould has now entered upon the stage

in which the formation of **apothecia** takes place. The spores germinate when they are set free, and the germ tubes grow into conidiophores.

In rainy seasons, when *Botrytis* attacks the grapes at a time when they are unripe, the mycelium, penetrating through the pulp, destroys the small amount of sugar in the grapes, and, as it kills the cells, a fresh immigration of sugar from the leaves is checked or rendered impossible. Such grapes act injuriously upon the quality of the wine. As the mycelium penetrates into the stalks also, causing them to die off, the very young grapes on such a cluster do not generally develop, but wither away. In years of good vintage the fungus does not usually appear until just before the grapes are gathered, and then gives rise to a different set of conditions. According to Müller-Thurgau's investigations the mycelium spreads principally through the skin of the grape, which becomes brown, leathery, and permeable to moisture. Thus in dry weather part of the water evaporates, the juice becomes more concentrated, and the grapes wither. The fungus does not penetrate far into the interior of the grape, but its growth affects both the acid and the saccharine constituents of the juice, and the must obtained from such grapes appears relatively richer in sugar and poorer in acids than is usual. Under favourable climatic conditions, especially in a dry atmosphere, white grapes which have been attacked by this "Edelfäule" can thus produce a must weak in acid, and hence yields a wine of finer quality and of special bouquet, due to some extent to the action of the fungus. This is particularly the case with varieties of vine which yield hard grapes with a high sugar and acid content—*e.g.*, the Riesling vine. A certain element of danger may, however, lurk in these attacks on the grapes, partly because *Botrytis* absorbs part of their albuminoids, rendering the must less nutritious for the yeast, so that the latter develops more slowly, and partly, as de Bary and, later, Behrens and Müller-Thurgau have shown, because the fungus secretes a poison which prevents the development of the yeast. Wines of this description, therefore, mature slowly, and are exposed to the attacks of foreign organisms. The fungus is invariably harmful to red and

blue grapes, even if they are attacked when ripe. Some species of *Botrytis* contain an enzyme which destroys cellulose.

It may be mentioned in conclusion that the leaves and stems of tobacco plants are subject to the attack of certain species of *Botrytis*, which bring about decay.

## 2. *Penicillium glaucum* (Fig. 33).

A mould which is far more widely distributed in the fermentation industries, especially in green malt, is *Penicillium glaucum*. It forms a felt-like mass on the substratum, at first white, then greenish or bluish-grey, and it spreads with great rapidity. The mycelium consists of transparent branched and divided filaments, which, when immersed in liquids, are liable to swell somewhat irregularly. From these filaments the conidiophores (*A*, Fig. 33) rise perpendicularly. They consist of elongated cylindrical cells, the terminal cell of which is soon arrested in its growth, and forms a thorn-like point ; the cell immediately below throws out one or more opposite branches, rising up close to the terminal cell, each consisting, like this, of a single pointed cell. In more vigorous individuals the branches may again ramify (compare *A*, upper half), or similar branches may also spring from the next cells, and these also ramify and become pointed as described above. In this tuft of branches each pointed cell (*sterigma*) breaks up into a series of spherical conidia, and finally the tuft carries a large number of conidia, arranged in series, which, when ripe, are readily scattered. These round, smooth conidia give to the patches of mould their greyish-blue colour ; when they fall upon moist surfaces, they are able to germinate at once. According to Cramer, they are very resistant to higher temperatures.

In culture experiments with this fungus, Brefeld made the interesting observation that *Penicillium* may occur under certain conditions with an entirely different form of growth. He enclosed cultures of this mould on slices of coarse, non-acidified bread, between glass plates, and allowed the culture to develop whilst excluding air as far as possible. Pairs of short thick branches then grow on to the mycelium, which

coil round each other (*B*, upper half); one part of this spiral throws out short, thick tubes (*C*), whilst the hyphal thread carrying the spiral develops numerous fine branches, which envelop it and form a covering (*D*), consisting of a dense inner and a felted outer layer; the inner cells are gradually coloured yellow, and the loose outer cells are cast off. In

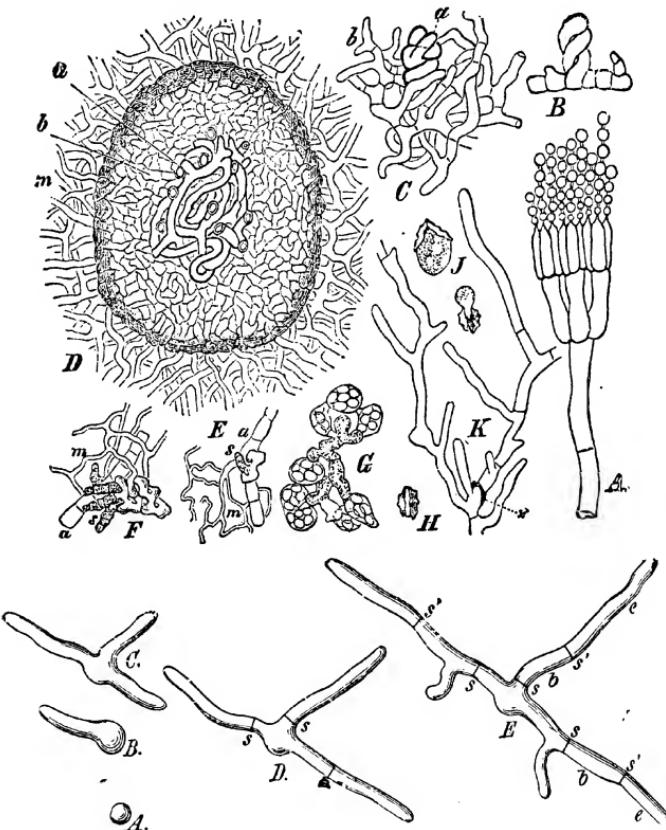


Fig. 33.—*Penicillium glaucum* (after Brefeld and Zopf).—*A*, Conidiophore; *B*, organs of generation; *C*, first development of the sclerotium (*a*, ascus-forming hyphae; *b*, sterile filaments); *D*, very young sclerotium in section (*a*, ascus-forming hyphae; *b*, sterile portion of the sclerotium; *m*, mycelium); *E* and *F*, ascus-forming hyphae; (*a*) with young asci (*s*) and sterile mycelium (*m*), threads (*m*) from a more developed sclerotium; *G*, group of ascospores with spores; *H*, spore; *I*, threads (*m*) from a germinating spore; *K*, young mycelium (with spore at *x*). *A-E* (below), germination of a germinating spore; *E*, young mycelium (with spore at *x*). *A-E* (below), germination of a conidium, after Zopf (more highly magnified): *A*, conidium before germination; *B*, it has thrown out a germ tube; *C*, three germ tubes have been formed; *D*, each germ tube shows towards the spore a transverse septum (*s*); *E*, each germ tube has become divided by another septum (*s'*) into a terminal cell (*e*) and an inner cell (*b*).

this small yellow ball a formation of swollen cells (E, F, G) gradually takes place by the continued branching of the spirals, and in each of these new cells eight large and lenticular spores are produced, which have a circular furrow on the margin, and three or four slight ridges on the outer membrane (exosporium). After the collapse and absorption of all the remaining contents the spores are set free, and the small yellow ball is then filled with the spore dust. The entire development requires six to eight weeks. The ascocarps may be preserved in a dry state for several years without losing their power of germination. When the spores (H) are sown, the exosporium bursts open like a shell at the circular furrow, and the endosporium swells and emerges (J), and elongates itself to a germ tube, which quickly develops conidiophores.

This fungus often causes dangerous diseases in wine. It develops freely in casks which have not been carefully cleaned, penetrating into the wood, and, in consequence of the decomposition caused by it, produces substances of disagreeable smell and taste, which subsequently diffuse into the wine. In moist seasons it forms a dense growth on grapes, attacks the sugar contents of the fruit, and brings about a peculiar decomposition. The mycelium seems to penetrate, not only into bruised, but also into sound grapes. They gradually acquire a yellowish-brown or greenish-yellow colour, and the fungus produces those well-known decomposition products which cause the mouldy taste in wine. The conidia of this fungus may exist for a long time in must or in wine upon which the germinating mycelium exerts a deleterious effect.

According to Wortmann, *Penicillium* has a particularly harmful effect on bottled wines. It penetrates the corks, thus giving rise to the corked flavour, and it may even grow right through the cork and develop in the wine, attacking some of its constituents, and rendering it turbid.

The name *P. glaucum* doubtless includes several distinct species, differing probably in their physiological character. A closely related species, *P. Roquefort*, occurs in Roquefort cheese. It is similar in appearance, but has larger conidia, and imparts a bitter flavour to the cheese. *P. Camembert* occurs in the cheese of the same name, in the ripening of which

the species plays a definite part. It forms a white growth (*P. album*) similar to that of *P. candidum* occurring in Brie cheese. At a later stage the colour changes to greyish-white or greyish-green. The last two species have large spherical conidia.

The same genus also includes *P. luteum*, with a yellow mycelium, changing to green or light brown when covered with conidia. The conidia are small and elliptical, and the sterigmata exceptionally long. This species forms yellowish-brown ascocarps; the ascospores are provided with prominent transverse stripes. It usually occurs on fruits, causing them to decay, but it may also occur on a variety of other substances. *P. italicum* seems only to affect lemons and similar exotic fruits, causing their decay. It forms a greenish-blue covering, and has ellipsoidal conidia somewhat larger in size than those of *P. luteum*. *P. olivaceum*, occurring on exotic fruits, and less frequently on others, has a distinct greenish-brown colour and large ellipsoidal conidia.

Most species of *Penicillium*, and especially *P. glaucum*, contain a number of different enzymes, one of which, a proteolytic enzyme, is present in all species hitherto examined. *P. glaucum* also possesses a diastase which, according to Laborde, converts starch into dextrin and dextrose. In addition, they contain invertase, maltase, a clotting enzyme, a casein-splitting enzyme, etc., and a poisonous substance which has not been identified. The species occurring in cheese react upon it by the secretion of an enzyme which splits up albumen.

Species of *Citromyces*, which have much the same structure as *Penicillium*, are able under suitable conditions of nutrient, with proper access of air and at the right temperature, to convert sugar into citric acid. The thin-walled conidiophores of these fungi are usually unbranched, sometimes have dilated ends, and possess a terminal tuft of sterigmata. The conidia are very small and spherical. The plants form a greenish covering like that of *Penicillium glaucum*, and are especially found on acid fruits and fruit juices.

Two species have been described by Wehner, *C. Pfefferianus*, forming a velvety coating, and *C. glaber* with an almost smooth surface; in other respects they appear to be almost identical.

Certain forms differing somewhat in character from the above were isolated in the author's laboratory. They have a technical value in the manufacture of citric acid, which is produced in a free state as the result of their activity. The fermentation is a form of oxidation, and is dependent upon free access of oxygen. For certain reasons, however, which are as yet unknown, the formation of citric acid is to be regarded as a progressive but imperfect oxidation. The quantity of acid can rise to 8 per cent. without exercising an appreciable effect on the vegetation, but beyond this point the acid is decomposed by the fungus. If, on the other hand, the acid is neutralised as fast as it is produced by the addition of chalk, the formation of acid continues. The difficulty that is encountered in carrying out this process on a large scale is due to the fact that a comparatively small infection with yeast or with *Penicillium glaucum* strongly affects the course of fermentation.

### 3. Aspergillus.

The most commonly occurring species is *Aspergillus glaucus* (Fig. 34), first fully described by de Bary. It forms a fine felty, greyish or greyish-green covering on various materials, and grows with great luxuriance on green malt.

The mycelium consists, as in the case of *Penicillium*, of fine transparent and branched threads, provided with transverse septa. Some of the hyphal threads grow up perpendicularly, are thicker than the rest, and are rarely branched or divided by septa. The upper ends swell to spherical flask-shaped heads (c), and these throw out from their entire upper portion radially divergent papillæ of an oblong form; these sterigmata (s) then develop at their apex small round protuberances, which are attached to the sterigmata by greatly constricted bases, and after some time break off to form independent cells (conidia). Below the base of the first conidium a second begins to form from the crown of the sterigma, and pushes the first upwards; a third then forms, and so on. Each sterigma thus carries a chain of conidia, the youngest of which lies closest to it. This occurs simultaneously over the whole surface of the enlarged end of the conidiophore,

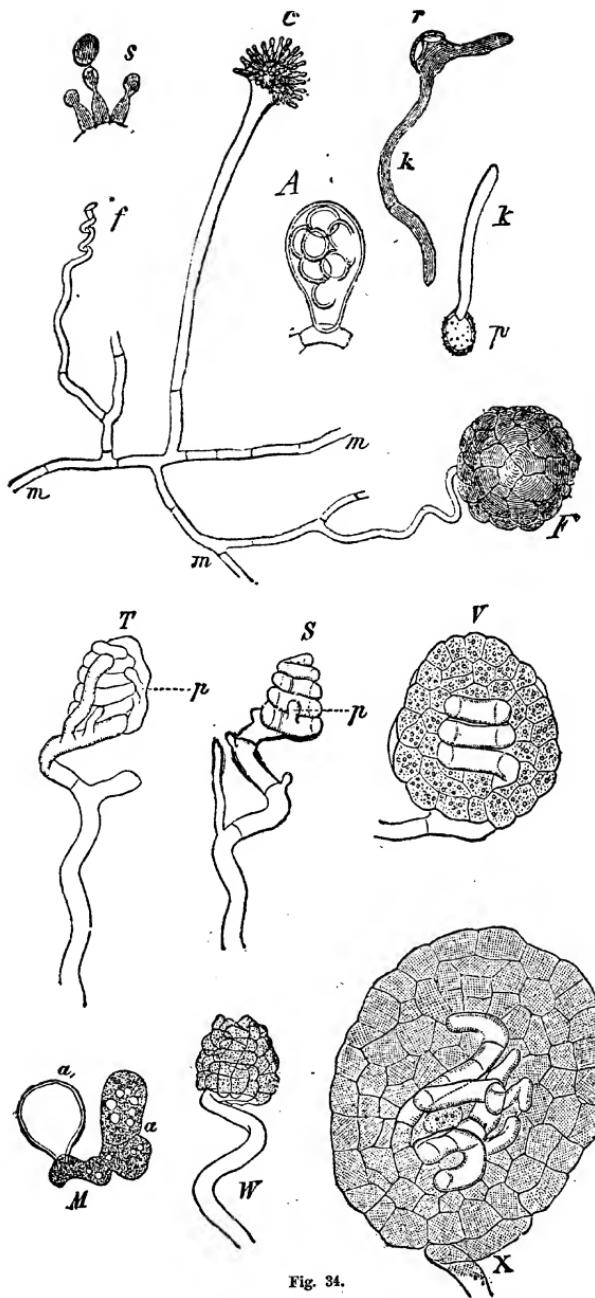


Fig. 34.

which is thus finally covered with a thick head of radially-arranged chains of conidia. These masses form the greyish-green dust which covers the mycelium.

Finally, the conidia separate, and then have a warty appearance on their outer surface. These small bodies can germinate (*p*) as soon as they are detached, and at once develop a new mould; upon this fact depends the rapidity with which the plant spreads. Under certain conditions, which are not yet sufficiently known, but in every case appear to be connected with a free supply of nutriment, the fungus develops perithecia. They appear at first as tender branches, which, at the termination of their longitudinal growth, begin to twine their free ends in the form of a spiral of four to six turns (*f*); the threads of the spiral gradually approach nearer together, until finally they come into contact, so that the whole end of

Fig. 34.—*Eurotium aspergillus glaucus* (de Bary).—*m*, *m*, hyphal thread, carrying a conidiophore *c* (from which the conidia have fallen), a peritheciun *F*, and the first rudiments of an ascogonium, *f* ( $\times 190$ ); *s*, three sterigmata, from the crown of a conidiophore, showing the conidia-constrictions; *p*, germinating conidium ( $\times 250-300$ ); *A*, Ascus; *r*, germinating ascospore; *k*, germ tubes; *S*, spiral ascogonium; at *p* the commencement of the growth of one of the enveloping hyphae; *T*, older stage; *W*, ascogonium, already surrounded by the envelope; *V*, longitudinal section of an older stage; in the centre the ascogonium, surrounded by the envelope, consisting of several layers; *X*, longitudinal section of a later stage of development; the ascogonium is enveloped in a sheath of many layers; it has loosened its convolutions, and is beginning to throw out the ascus-forming branches; *M*, portion of an older ascus-bearing branch; *a*, a young ascus; *a*<sub>1</sub>, an older ascus which has burst.

the filament takes the form of a helix (the ascogonium). Two or more small branches which cling closely to the spiral then grow from the lowest turn of the helix. One of these (*S*, *T*, *p*) quickly outstrips the others in growth; its upper extremity reaches the uppermost turn of the helix, and fuses with it. The other branch or branches likewise grow upwards along the spiral, shoot out into new branches, and gradually become so interlaced that the spiral is finally surrounded by an unbroken envelope (*W*). These branches divide slowly into septa perpendicular to the surface, and the envelope consequently consists of short, angular cells, in which new septa appear parallel to the surface, so that the envelope thickens and is composed of many layers (*V*, *X*, *F*). The small sphere now formed is about 0.25 mm. in diameter; the outermost layer is at first yellow and then brown; the inner layers remain soft, and are finally dissolved. After a time the spiral

extends and throws out on all sides branched filaments, which dislodge the inner layers of the envelope. These branches finally take the form of an ascus (*M* and *A*), eight spores being formed in each. After the breaking up of the ascii the spores lie loose in the interior of the peritheciun, and are liberated by the rupture of its fragile wall. The spores are bi-convex, and carry a longitudinal furrow; they possess an opaque outer membrane and an inner one, which on germination bursts the outer membrane, forming two valves (*r*). This species thrives best at a temperature of about 25° C. The enzymes that are associated with *Aspergillus glaucus* are, amongst others, diastase, invertase, maltase, and a proteolytic enzyme.

Another well-defined form is *A. flavus*, with a yellowish-green mycelium occurring frequently on bread, and also on dry excrement. Its optimum temperature is at 37° C., and it is believed to be pathogenic, its presence having been detected in the human ear. Its conidia are usually smooth.

*A. fumigatus* occurs on very different substrata, and is also pathogenic both to man and beast. It forms a greyish or greyish-green vegetation, and has an optimum temperature at 40° C. It produces minute conidiophores with small spherical conidia and brown perithecia. It contains the same enzymes as those occurring in *A. glaucus*. It plays a part in the spontaneous ignition of dry vegetation, and may, according to Cohn, cause a rapid rise of temperature in green malt.

A particularly interesting form of *Aspergillus* is *A. (Sterig-matocystis) niger*, which produces branched sterigmata. It is of very general occurrence, and forms blackish-brown patches of conidiophores together with spherical, smooth or warty conidia. It also produces yellowish sclerotia (without spores). Its optimum is at 40° C. It grows well in an extract of oak galls, and in a tannic acid solution. It is of technical importance, as it is used in the preparation of gallic acid from tannin. According to Fernbach and Pottevin, a special enzyme secreted by the fungus is active in this process. It is produced only on substances containing tannic acid, and has its optimum at 67° C. Wehmer has shown, moreover, that when grown on carbohydrates, such as grape sugar, *A.*

*niger* forms free oxalic acid, and that if a temperature of 15° to 20° is maintained, especially in presence of calcium carbonate, a considerable amount of acid is produced. Like other *Aspergillus* species, it contains diastase, invertase, maltase, and a proteolytic enzyme. A large number of other enzymes have been identified in this species, which has been thoroughly investigated in its physiological relationships.

In the preparation of the strongly fermented Japanese rice wine (saké), *Aspergillus Oryzae* is systematically employed. This fungus forms yellowish-green patches, and grows on the most varied media. The conidiophores terminate in spherical or club-shaped swellings, and the sterigmata radiate either from the upper portion only, or from the entire surface of the swollen end of the conidiophore. The conidia are large and yellowish-green; they are either oval or spherical, and may be either smooth or covered with fine warts. According to Wehmer, they can maintain their vitality for many years, a fact which has been substantiated by observations made in the author's laboratory. The existence of a yeast stage of this fungus, due to the budding of the conidia, has also been confirmed by the author's direct observation.

Rice grains, freed from their hulls, are steamed, but the aggregation and gelatinisation of the grains must be avoided. In order to prepare a malt serviceable for brewing from these grains, which are unable to germinate or to exercise the usual diastatic activity, the mass of grain is mixed with other rice grains, which are coated over with the mycelium and conidiophores of *Aspergillus Oryzae*, or the yellowish-green spores ("Tane-Koji") of the fungus are mixed with the steamed rice grains. In moist and warm air, after the lapse of about three days, a white velvety mycelium is developed on the rice, which imparts to the mass an agreeable odour resembling apples or pineapples. Before the fructification of the fungus takes place, a fresh quantity of steamed rice is introduced, and this also is gradually coated over with mycelium; the process is repeated several times. In the "koji" mass thus produced, a part of the starch has been hydrolysed, and some of the albuminoids have been rendered soluble. The koji mass is mashed in the cold, 21 parts of koji being

mixed with 68 parts of steamed rice and 72 of water. This pasty mass ("Moto") is allowed to remain at about 20° C.; after some days it clarifies. The conversion of starch and dextrin into sugars progresses, and at the same time a spontaneous and very violent alcoholic and lactic fermentation sets in. In this fermentation there occurs a *Saccharomyces* which is able to produce a very high percentage of alcohol. The mass is now warmed to about 30° C. At the end of two or three weeks the primary fermentation is finished. The product, after being filtered, is subjected to a secondary fermentation, and the liquid is then clear and yellow, like sherry, containing 13 to 14 per cent. of alcohol. It is usually pasteurised at 50°-70° C. in iron vessels.

According to Kellner, *Aspergillus Oryzae* also plays an important part in the preparation of Japanese Shoyu or Soja. Saito's exhaustive biological researches into the method of preparation have shown that a koji is used consisting of a growth of the fungus on a mixture of boiled soja beans and parched wheatmeal. The koji is mixed with salt and water at 45° C., and then allowed to ferment, a process which may last a year. The mass gradually acquires a rich reddish-brown colour, and an aromatic odour. It is then placed in small sacks and pressed, an almost clear liquid exuding, which is then further clarified and pasteurised at 50°. In this fermentation lactic acid and alcohol are formed, and a number of dissolved aromatic substances. Along with *A. Oryzae*, Saito found a large number of other organisms taking part in this fermentation, including a new yeast species, *Saccharomyces Soya*, which is specially active in the production of alcohol. The lactic acid is formed by two bacteria, *B. Soya* and *Sarcina Hamaguchiae*. According to Kellner, *Aspergillus Oryzae* is also of importance in the preparation of the Japanese bean mash (Miso).

Korschelt found that the hyphæ of this species secrete a diastase, which, like malt diastase, converts starch into dextrin and maltose, an observation confirmed by Atkinson, and subsequently by Cohn and Büsgen.

Atkinson found an enzyme in koji which is soluble in water, inverts cane sugar, and converts maltose, dextrin, and starch-

paste into dextrose. The researches of Kellner, Mori, Nagaoka, and Okumura have likewise shown that the koji mass possesses a strongly invertive enzyme, which converts cane sugar into dextrose and laevulose, maltose into dextrose, and starch into dextrin, maltose, and dextrose. The various micro-organisms which occur in the koji mass probably contain these different enzymes. Saito observed a peculiar kind of acid formation due to this ferment.

In Java, the *Aspergillus Wentii*, described by Wehmer, is used for the preparation of Chinese soja, and the "Tao-Tjiung" (bean mash). It occurs spontaneously on soja beans. It forms a snow-white mycelium, coloured brown at a later stage by the globular conidia, which exhibit a fine warty structure; the sterigmata are not ramified. According to Prinsen Geerligs, who described the technical application of the fungus, it not only possesses a peptonising and diastatic ferment, but is also able partially to dissolve the cell-walls of the soja bean. When the boiled soja beans have been sufficiently acted on by the fungus, they are mixed with a concentrated salt solution, after which the mixture is boiled along with sugar and various aromatic herbs. The process is, therefore, not one of fermentation.

Saito has investigated the preparation of the Japanese yam brandy, involving the use of a special kind of *Aspergillus* (*A. Batatae*), by means of which the starch of the yam tubers is converted into sugar. This species first forms yellowish-green, changing to dark brown, patches, consisting of brown, spherical, and finely grained conidia. It possesses the same enzymes as the other species of *Aspergillus*. The alcoholic fermentation is brought about by a special form of yeast, *S. Batatae*.

#### 4. Mucor.

The genus *Mucor* belongs to the most interesting group of moulds with which we have to deal, since it embraces species with marked fermentative activity and great power of converting starch into sugar. They occur as grey or brown, felt-like masses, often attaining a considerable height—occasionally measuring several inches—in which small yellow,

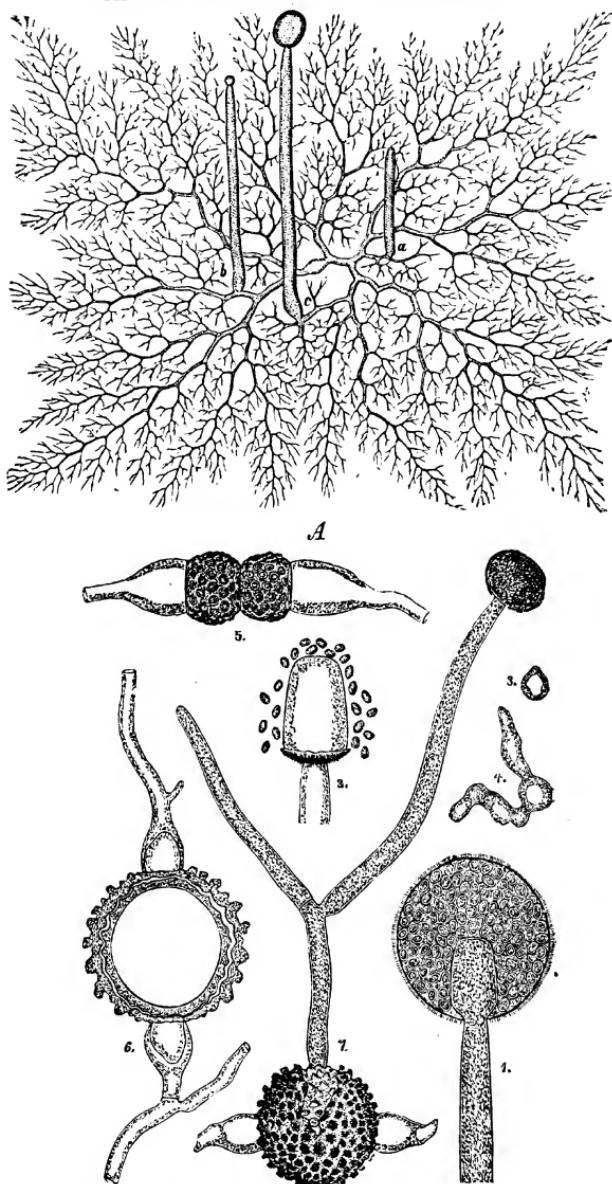


Fig. 35.—*Mucor Mucedo* (after Brefeld and Kny).—A, tree-like ramified mycelium with isolated thicker upright branches (a, b, c); 1, Sporangium; 2, columella and spores; 3, 4, germinating spores; 5, 6, development of the zygospore; 7, germinating zygospore with sporangium.

brown, or black globules may be distinguished by the naked eye. Only the more commonly occurring species are described.\*

*Mucor Mucedo* (Fig. 35), one of the most beautiful mould growths, and one which occurs very generally on the excreta of herbivorous animals and especially on horse excreta, has a transparent white mycelium, which develops numerous and delicate ramifications both above and below the surface of the substratum. In its earliest stages of development, and until the sporangia begin to form, it is without transverse septa, and, therefore, unicellular. Single vigorous branches, the sporangiophores, rise from the mycelium; the points of these branches which, according to Zopf, contain a reddish-yellow fatty colouring matter, swell greatly, the protoplasm withdraws from the stalk into the enlarged heads, and below the swelling a transverse septum is finally formed whereby the sporangium is cut off from the sporangiophore. The transverse wall arches upwards, and forms a short column (columella) in the interior of the spherical head, whereby an inner space of peculiar form (1) results. The protoplasm of this space breaks up into a number of small portions, which are gradually surrounded by a membrane and rounded off; these are the spores. At the same time the sporangium is coated on its outer surface with small needle-shaped crystals of calcium oxalate. As soon as the ripe greyish-brown sporangium takes up moisture, the wall dissolves, and the spores with their yellowish contents are scattered on all sides along with the swelling contents of the sporangium. The columella, which projected upwards in the sporangium, still remains at the end of the sporangiophore; this is now surrounded at its base by a collar (2), the remains of the outer wall of the sporangium. When the refractive spores fall on a favourable substratum, they swell very considerably, and send out one or two germ tubes (3, 4), which quickly develop to a vigorous mycelium. The optimum for growth lies between 20° and 25° C.

In addition to this mode of reproduction, *Mucor Mucedo* and the other species possess a sexual method of reproduction, which takes place by means of a conjugation of two branches of the same mycelium. Two such short branches, filled with

\* A systematic description has been given by Fischer and von Schroeter.

protoplasm, and growing towards each other, form club-like swellings, and come in contact at their free ends, which flatten out (5). Each of the branches is then divided into two cells by a septum, and the end cells, which are in contact (the conjugating cells), coalesce by dissolution of the original double wall which separated them. The two conjugating cells are either equal in size, as in *Mucor Mucedo*, or unequal, as in *Mucor stolonifer*. The new cell thus formed—the zygospore (6)—quickly increases in size and expands to the shape of a ball (in *Mucor stolonifer* to the shape of a barrel), after which the wall thickens, and becomes stratified; externally, it is dark in colour and covered with wart-like excrescences. These outer layers are very resistant to the action of acids. The contents possess an abundance of reserve substance (fat). Cases occur where the zygospore develops from a single cell without conjugation, and occasionally such a cell is formed at the tip of one of the mycelial hyphæ. The zygospores are generally only able to germinate after a long period of rest; the germ tube, after bursting the outer layers, quickly develops sporangia as described above (7). Thus, in the zygospore we find a resting stage of the plant, an organ which by its structure enables the mould to preserve life during periods unfavourable to growth.

These spores are usually formed only on the surface of the substratum with free access of air.

The conditions on which the formation of zygospores depend have been exhaustively investigated by Blakeslee, who has shown that, in the case of the majority of species examined, it was essential that the conjugating hyphæ should belong to different individuals. In the case of *M. Mucedo* it is necessary that these individuals should be derived from spores originating from different sporangia, otherwise no zygospores are formed. In some species a difference could be detected in the structure of the two individuals which formed the zygospores. In a minority of cases the zygospores were, however, formed from one and the same mycelium. Temperature affects their formation; thus in *M. Mucedo* they were produced at ordinary room temperature, but not at 26°-28° C.

*Mucor racemosus* (Fig. 36), which occurs on bread and decaying vegetable matter in very variable forms, has a branched, multicellular sporangiophore, which may also attain to a considerable height. Like *M. Mucedo*, the optimum temperature ranges from 20°-25° C. The brownish sporangia are developed at the ends of the branches. The spores are colourless. Both the aerial and the submerged portions of the mycelium are capable of forming transverse septa, dividing the hyphae into a number of short cells. These are

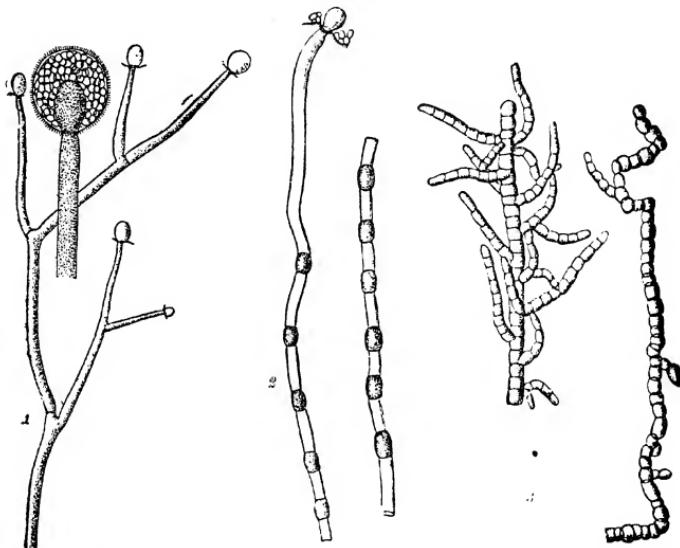


Fig. 36.—*Mucor racemosus*.—1, Branched sporangiophore and sporangia (highly magnified); 2, hyphae with chlamydospores; 3, branched mycelium; gemmae formation.

usually filled with protoplasm, and assume a spherical or barrel shape; this was first observed by Bail. They are termed **gemmae**. The cells frequently form thickened cell-walls, and store up reserve food material, thus constituting a resting spore (**chlamydospore**). Both kinds of cells after separating from the mycelium may again vegetate under suitable conditions.

When free access is given to atmospheric oxygen, both spores and gemmae germinate and from an initial germinal

hypha develop into a mycelium. The case is different, however, in the absence of oxygen. Hansen has shown by experiment that under these conditions, not only the spores and gemmæ, but even the normal mycelial hyphae develop yeast-like budding cells, and thus form the "mucor yeast" or "spherical yeast." The carbon dioxide formed by the fungus is only of value in the formation of the yeast stage by excluding oxygen. Whilst the absence of oxygen is a general condition governing the formation of yeast cells from mucor, there are a few species which demand the presence of sugar. *Mucor racemosus* requires sugar, whilst *Mucor alpinus* (discovered by Hansen) does not require it. By cultivating *M. racemosus* in a flask completely filled with wort, through which a stream of carbon dioxide is passed, a growth consisting exclusively of mucor yeast can be obtained.

*Mucor erectus*, with greyish-yellow transparent sporangia, which may be found, for instance, on decaying potatoes, has the same microscopic appearance as *Mucor racemosus*; physiologically, however, it differs from this species.

*Mucor circinelloides* (Fig. 37) has a very characteristic appearance. The mycelium (1) shows the remarkable branching which occurs in some of the species of *Mucor*. The main branches (b) send out short, root-like branches (c) with frequent forking; at the base of these come new mycelial branches (r), which grow erect, and are able to form sporangia (2 to 5); the sporangiophore is sympodially branched. During its development it curls up, and to this fact the species owes its name of *circinelloides*. In this form, as well as *Mucor spinosus*, the mycelium, when submerged in a saccharine liquid, produces gemmæ, similar in formation to those of *Mucor racemosus* and *Mucor erectus*. *Mucor spinosus* has a greyish-blue mycelium with spherical spores and brownish-black sporangia, which is distinguished by the uppermost part of the columella being studded with pointed, thorn-like protuberances.

Finally, *M. alternans* belongs to this group, and bears a similarity to *M. circinelloides*. This fungus has the distinction of being the first of the *Mucor* species which was shown by Gayon and Dubourg in 1887 to possess the property of fermenting dextrin.

The most interesting of all the species of *Mucor* is *M. (Amylomyces) Rouxii*, on account of the great use which is made of it for the conversion of starch into sugar on a commercial scale. It was isolated by Calmette in 1892 from "Chinese yeast," small greyish-white cakes, which consist of rice grains kneaded together with different kinds of spice. Calmette, however, only described the characteristic mycelium which exhibited gemmæ (chlamydospores), and called the

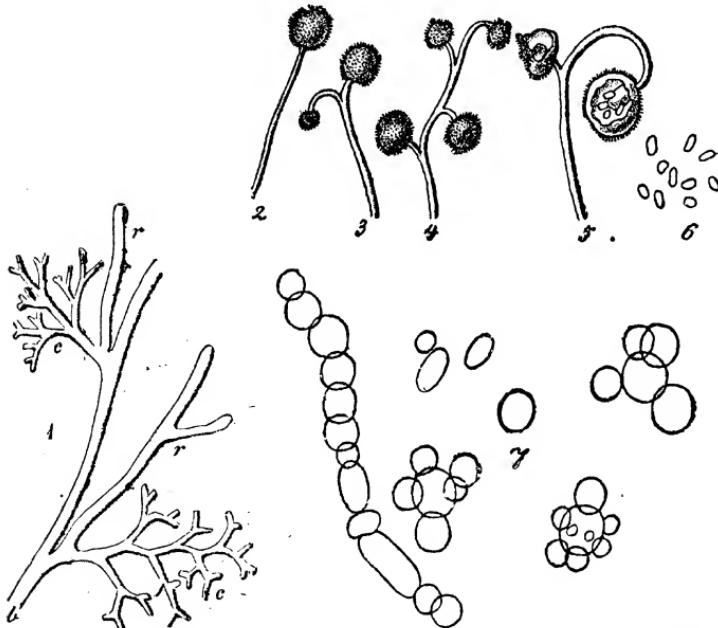


Fig. 37.—*Mucor circinelloides* (after van Tieghem and Gayon).—1, Mycelium; *b*, main branch; *c*, root-like branches; *r*, axillary branches; 2-4, development of sporangia; 5, opened sporangia; 6, spores; 7, submerged mycelium and budding cells.

fungus *Amylomyces*. Wehmer subsequently described the sporangia, and Vuillemin described in detail its characteristic features. On a solid substratum, such as rice, it forms a yellow covering, which is due to an aggregation of yellow oil in the cells. The same appearance is found when the mycelium spreads over the surface of liquids, but the submerged portions are grey in colour. A temperature slightly above 30° C. is

best suited to its development. The mycelium frequently remains sterile. The fructifying hyphae are of very varying length, the sporangia small, almost spherical, and both they and the elliptical spores may be either light or dark in colour. The fungus forms both gemmæ and "spherical yeast." Like most other species of *Mucor*, it has a tendency to vary in shape.

Another species, *M. Praini*, with similar characters to the foregoing, was isolated by Nechitch from Indian rice cakes. It has spherical sporangia, yellow or dark brown in colour, and colourless spores of varying shape.

A third species, isolated from Javan rice cakes, *M. javanicus*, has been described by Wehmer. It forms a yellow growth on rice, produces a raised cushion of sporangia, yellowish-grey or light brown in colour. The sporangia are small, yellowish-brown, and transparent, whilst the spores are colourless, and of irregular shape. Like the former species, it is able to convert starch into sugar, and to bring about alcoholic fermentation.

One of the most widely distributed members of the genus, but differing considerably in form from the species already described, is *Rhizopus nigricans* (formerly known as *M. stolonifer*), Fig. 38. The species attains a considerable size, and occurs very commonly on succulent fruits. This mould is easily recognised, for its brownish-yellow mycelium shoots out diagonally with thick hyphae without septa. These attain a length of about 1 cm., and then droop until their points touch the surface of the substratum, and then send out fine ramified hyphae resembling rootlets into the latter, whilst other hyphae rise perpendicularly and develop sporangia; finally other branches form new runners. The black spherical sporangium possesses a high, dome-shaped columella, which is contiguous with the broadened end of the sporangiophore, and develops a number of dark brown spores, round or angular. When these are freed by the absorption of the sporangium wall, the columella curves over on the sporangiophore like an umbrella, the line of junction of the external wall remaining in evidence in the form of a collar. In this species no formation of gemmæ has been observed. Zygospores are produced by the fusion of hyphae, which, according to Blakeslee, belong

to different mycelia. *Rh. nigricans* occurs on a great variety of juicy fruits, causing them to decay, and thus working considerable havoc. Behrens has shown that the damage is caused through the secretion by the fungus of a poisonous substance, which kills the fruit cells. It is also of frequent occurrence on malt.

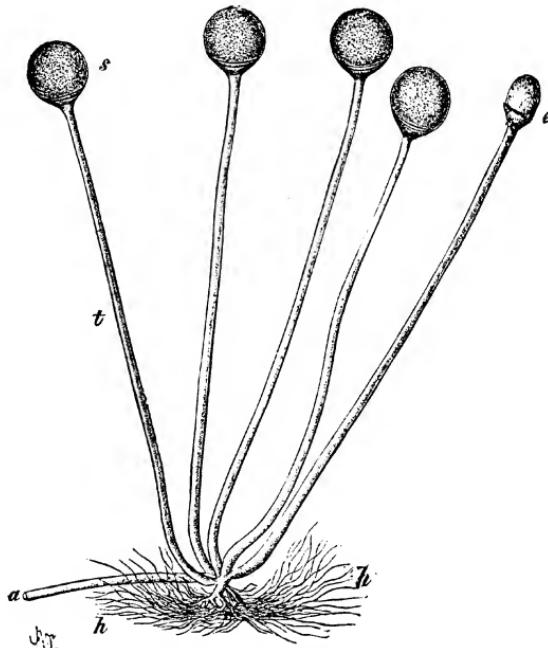


Fig. 38.—*Rhizopus nigricans* (after Brefeld).—*a*, End of a runner or stolon; *t*, sporangiophore; *s*, sporangium; *e*, columella; *h*, root-like hyphae or rhizoids.

A similar species, *Rh. Oryzae* (*Chlamydomucor Oryzae*) was, discovered on Javan rice cakes by Went and Prinsen Geerligs. On account of its power of dissolving starch, it is employed in the preparation of arrack from rice. It produces large numbers of gemmæ. A specially interesting form is *Rh. japonicus*, which, like *M. Rouxii*, is applied industrially for the conversion of starch, especially of maize starch, into sugar. It was isolated by Boidin in 1900 from Japanese koji, and, like the species discovered by Calmette, was called *Amylomyces* ( $\beta$ );

it was described more exactly by Vuillemin. It shows a great resemblance to both the former species, and, like *Rh. Oryzae*, it forms gemmæ. Vuillemin also described *Rh. tonkinensis*

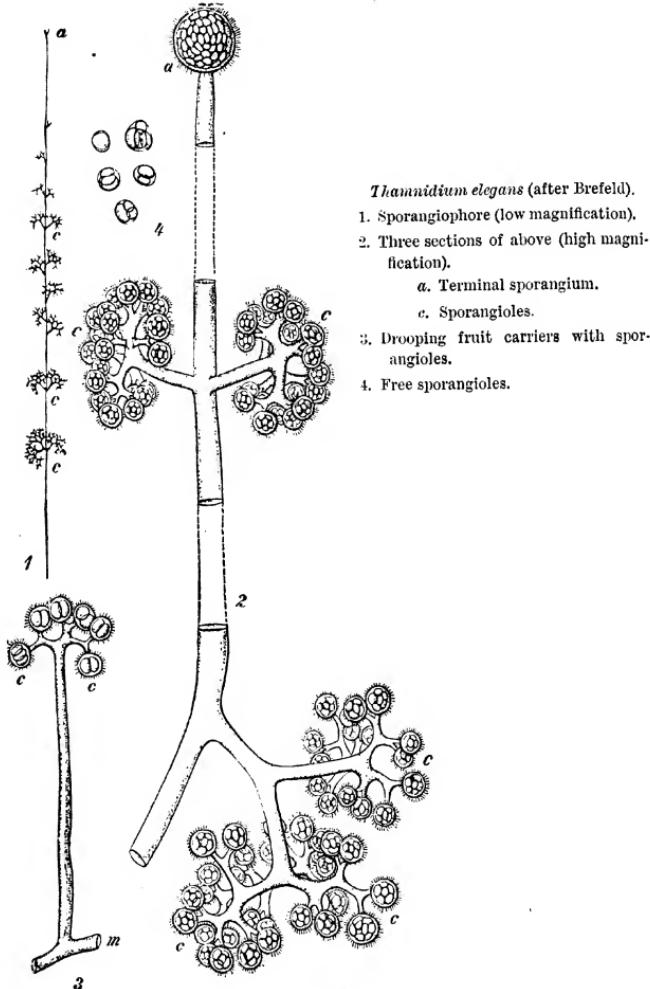


Fig. 39.

(*Amylomyces*  $\gamma$ ), which has the same structure as the foregoing, but shows a different behaviour towards the sugars.

Amongst other moulds standing in close relationship to the *Mucor* species, *Phycomyces nitens* is frequently referred to in the literature of this subject. It usually occurs on oily substrata, but also on bread, excrement, etc. It resembles *Mucor*, and its olive green sporangiophores with their metallic lustre attain to an extraordinary size. The sporangium is black, the columella pear-shaped, and the spores yellowish.

*Thamnidium elegans* (Fig. 39) frequently occurring on the dung of various animals, on bread, etc., is a fine mould with sporangia recalling those of *Mucor*; but in addition to the terminal sporangium formed at the apex of the main sporangiophore, the latter gives rise to a number of forked side branches, on which sessile sporangia (sporangioles) are formed without columella and with fewer spores.

In conclusion, we may allude to *Sporodinia grandis*, a fungus often met with on toadstools growing in woods. It forms a dense felt of branching hyphae with numerous sporangia, and also produces zygospores.

Hansen's determination of the limits of temperature for the three species, *M. racemosus*, *neglectus*, and *alpinus*, when grown on wort-agar gelatine and in wort, proved that the formation of sporangia and zygospores can go on at a slightly lower maximum than is required for vegetative growth (the behaviour is thus analogous to that of the Saccharomycetes). The development of sporangia can, however, go on at the same minimum as that required for vegetative growth. The species vary with regard to the temperature limits for sporangia, on the one hand, and for zygospores, on the other; thus *M. alpinus* exhibits a higher maximum for the formation of sporangia than of zygospores, but the reverse is the case with *M. neglectus*. It follows that the temperature limits may serve to determine the species. *Mucor racemosus*, for example, when grown on the media alluded to, gives as limits for vegetative growth a maximum of 32°-33° C., and a minimum of 0.5° C.; *M. alpinus* a maximum of 29°-31° C., and a minimum of 0.5° C.; *M. neglectus*, maximum 33° C., and minimum 3° C.

The *Mucor* species are of special interest to us, because they can act, in varying degrees, as true alcoholic ferment.

Their fermentative power is not connected with the formation of budding gemmæ, for these have not been observed in either *Mucor Mucedo* or *M. stolonifer*. This form of fermentation has been regarded as a special kind of breathing. Unlike normal respiration, such as is performed by every organised being—the absorption of oxygen and exhalation of carbon dioxide—it can take place in the absence of free oxygen. The oxygen in the cell contents makes fresh intra-molecular linkings, with the result that the carbohydrates, and more particularly the sugars, become disintegrated, so that not only carbonic acid but also alcohol is produced. Adopting the term suggested by Pflüger, the process is known as intramolecular respiration. This conception implies that the fermentative change produced by *Mucor*, which is only possible in the absence of free oxygen, differs essentially from that brought about by yeast, which can go on either in the presence or absence of free oxygen. Wehmer's experiments with two species of *Mucor* (*M. racemosus* and *M. japonicus*) have shown, however, that the production of alcohol was not diminished by the constant bubbling of air through the liquid, nor yet when the fermentation is carried out in very thin films of liquid with a large exposed surface. In other directions the two kinds of fermentation possess characteristics in common, and the collective evidence makes it difficult to regard the processes as essentially different. On the other hand, Palladin and Kostytschew and others have proved that the two fermentations are not identical. A special alcohol enzyme, such as that isolated from yeast, has not been isolated from *Mucor* mycelium. Kostytschew observed, however, that the mycelium of *M. racemosus*, which had been killed by treatment with acetone, was able to produce an amount of carbon dioxide equal to that evolved by the living cells. *M. racemosus*, Hansen's *M. neglectus* and (according to Saito) *Rhizopus japonicus*, var. *angulosporus*, and *Rh. Tamari* are the only species capable of inverting and fermenting a cane-sugar solution. This was proved by Fitz for *M. racemosus*, and confirmed by Hansen and others. The great majority of species are, however, able to ferment maltose, invert sugar, and dextrose.

Considerable diversity may be observed amongst the

different species in regard to the production of alcohol. The same rules which govern yeast fermentations seem on the whole to apply to these processes. Thus, according to Wehmer, when the general conditions are favourable, in presence of oxygen and at a medium temperature, the fermentation is practically completed in the course of a few days. A remarkable feature of the fermentation produced by these fungi is that the liquid remains clear throughout the operation.

Some of the results obtained during Hansen's investigations may be quoted to show the difference in the productivity of the various species.

*M. erector* possesses the greatest fermentative activity. In beer-wort of ordinary concentration (14°-15° Balling), it yields up to 8 per cent. by volume of alcohol. It also induces alcoholic fermentation in dextrin solutions, and converts starch into reducing sugar. *Mucor spinosus* yields up to 5.5 per cent. by volume of alcohol in beer-wort. In maltose solutions distinct fermentation phenomena were observed, and after the lapse of eight months the liquid contained 3.4 per cent. of alcohol. *Mucor Mucedo* has a comparatively feeble fermentative power both in wort (up to 3 per cent. of alcohol) and in maltose and dextrose solutions. *Mucor racemosus* produces as much as 7 per cent. of alcohol in wort, develops invertase, and ferments the inverted cane sugar; thus, like the two species above mentioned, it occupies a particular position.

According to Gayon, *Mucor circinelloides* exercises a very powerful action on invert sugar (yielding 5.5 per cent. by volume of alcohol). According to Wehmer, *M. javanicus* produces 4 to 5 per cent. of alcohol in a few days.

Whilst the *Mucor* species are of no technical importance as alcohol producers, those possessing powerful diastatic enzymes, capable of converting starch into sugar, occupy an important place in industry. It has already been mentioned that they have been used by the Asiatic races for centuries. Their systematic use in Europe began in 1892, when Calmette isolated *M. Rouxii* from "Chinese yeast." The diastatic enzyme of this fungus reacts most powerfully at 35°-38° C. and produces chiefly dextrose. The process, carried on as it

now is on a large scale in special factories, consists in first boiling the starch (maize or rice starch) under pressure, then liquefying the mass by the addition of a small quantity of green malt, or hydrochloric acid, and sterilising the fluid at a high temperature. By adding a culture of the fungus grown from spores at 38° C. the change into sugar is soon effected.

In 1895 Went and Prinsen Geerligs published their research on *Rhizopus Oryzae* (*Chlamydomucor Oryzae*), which likewise converts starch into dextrose.

Shortly afterwards Collette and Boidin announced the discovery of two similar species, *Rhizopus tonkinensis* and *Rh. japonicus*, which react more vigorously than *M. Rouxii*; other species have subsequently been discovered.

A number of *Mucor* species produce small quantities of acid in sugar solutions. A detailed investigation of this subject has not yet been carried out. Wehmer, however, has observed the formation of citric acid by *M. pyriformis*, and several species have been shown to produce oxalic acid. Most species liquefy gelatine, but quite slowly as a rule. Albumen-splitting enzymes occur in the different species, and some of these appear to play a part in the ripening of cheese.

##### 5. *Monilia* (Figs. 40 and 41).

A number of different fungi of comparatively simple structure are described under this name in works on mycology. From a mycelium, the colour of which varies according to the species, branches are thrown up which give rise to series of oval or elliptical spores. The genus has an interest for us on account of one of its species, named by Hansen *Monilia candida* from Bonorden's description, which possesses very remarkable physiological properties. It occurs in nature in the form of a white layer covering fresh cow-dung, and on sweet, succulent fruits. When introduced into wort, it develops a copious growth of yeast-like cells. At the same time it excites a vigorous alcoholic fermentation, and whilst this is progressing forms a mycoderma-like film on the liquid; the cells in this film extend further and further, and finally

form a complete mycelium. During the early fermentation the fungus produced only 1·1 per cent. by volume of alcohol, whilst *S. cerevisiae* gave 6 per cent.; but the *Monilia* continued the fermentation, and produced at the end of six months 5 per cent. by volume of alcohol, whilst the culture yeast gave no further quantity.

Hansen states that *Monilia* does not secrete invertase, but, nevertheless, ferments cane sugar, from which he concludes that cane sugar is directly fermentable. He suggests,

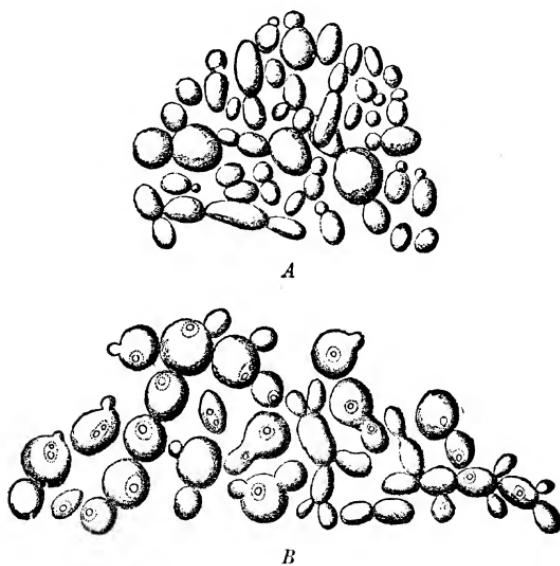


Fig. 40.—*Monilia candida* (after Hansen).—A, growth in beer-wort or other saccharine nutritive liquids; B, cells of a young film-formation.

however, the possibility that cane sugar may be converted into invert sugar in the interior of the cells, and that the latter is immediately fermented.

Hansen's observations were confirmed by the work of E. Fischer and P. Lindner, and subsequently by Buchner and Meisenheimer. They proved that an inverting enzyme cannot be extracted either from the fresh or from the dried vegetation.

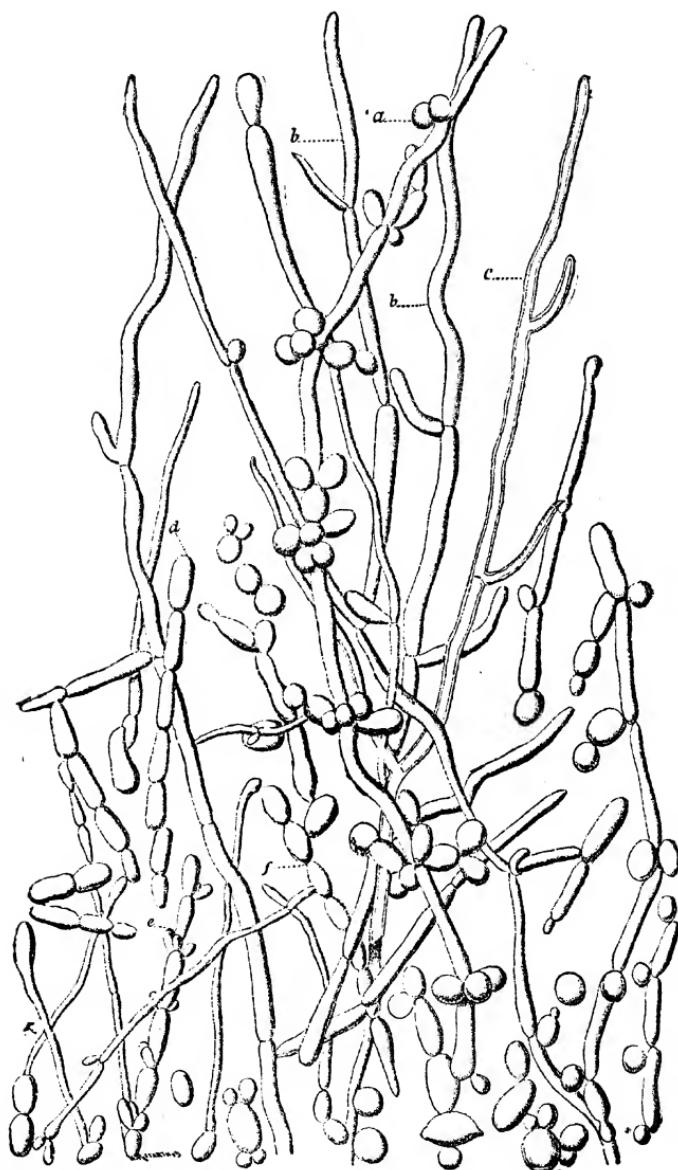


Fig. 41.

On the other hand, they were able to obtain preparations which inverted cane sugar actively either by using the dried fungus, by grinding the cells with powdered glass, by killing them with acetone, or by pressing out the juice (see Chap. v.). Thus the fungus contains an inverting enzyme, but it is completely retained by the protoplasm of the living cell. In contrast with yeast invertase, *Monilia* invertase is insoluble in water; it does not diffuse, as yeast invertase does, through the cell-wall, or through the protoplasmic lining of the cells, neither does it diffuse through parchment. In this respect, and in the ease with which it is decomposed, it possesses characters in common with Buchner's zymase (see Chap. v.).

According to Fischer, maltose is split up both by fresh and by dried *Monilia*, and also by an aqueous extract of a dried growth; he, therefore, infers that *Monilia* contains the enzyme maltase recently discovered by him in *S. cerevisiae*.

Fig. 41.—*Monilia candida* (after Hansen).—Mould growths like *a* are frequent; they consist of chains of elongated cells, more or less thread-like, and rather loosely united; at each joint there is generally a verticil of oval cells, which readily fall off; *b* represents another form, also of frequent occurrence, but distinguished from the former by having no verticillate cells; instead of these there generally issues from every joint a branch of the same form as the mother cell, but shorter; the links of these chains are often closely united, the constrictions in many cases disappear, and a very typical mycelium, with distinct transverse septa (*c*) is produced; the forms *b* and *c* occur in the nutritive medium, *a* commonly on the surface. Forms like *d* have much resemblance to *Oidium lactis*. *e* shows a train of pear-shaped cells with verticillate yeast-cells resembling *S. exigua*; the chain of lemon-shaped cells represented at *f* closely resembles Ehrenberg's figures of *Oidium fructigenum*. Between the principal forms described there are numerous yeast-cells of different forms, variously arranged in colonies.

According to Bau, *Monilia* also ferments dextrin formed from diastase.

As recently as 1883, *Monilia candida* was the only fungus known to be capable of fermenting cane sugar, although not secreting invertase. Since then Zopf, Beijerinck, Behrens, and other investigators have observed this phenomenon in the case of a few other micro-organisms; they form, however, rare exceptions. It forms another example of the unexpected gradations that exist in nature.

A certain amount of carbon dioxide and ethyl alcohol is developed in liquids undergoing a *Monilia* fermentation.

Finally, this fungus is distinguished by its power of withstanding high temperatures. In beer-wort and cane-sugar solutions it develops vigorously at 40° C., and induces an active fermentation at this temperature. The limits of

temperature for the development of *Monilia* in wort are, according to Hansen, maximum 42°-43° C., and minimum 4°-6° C.

Many other species have been described, amongst which may be mentioned *M. sitophila*, discovered by Went, which grows on the earth nut (*Arachis hypogaea*) in West Java. Its mycelium extends by degrees throughout the entire fruit, the hyphæ assuming a yellow colour on exposure to air. By means of the various enzymes which the fungus contains, a change is brought about in the fruit contents. In this fermented condition the earth nuts are eaten in large quantity by the natives. *Sachsia suareolens*, discovered by P. Lindner, is also an interesting fungus belonging to this group. It produces a high percentage of alcohol in wort, and develops a wine bouquet.

#### 6. *Oidium lactis* (Fig. 42).

*Oidium lactis* is a mould which has played an important part in the literature of the physiology of fermentation, and in that of medicine. It is known as the milk mould.

Some authors have sought to establish the theory that this fungus is a stage in the development of species which, under other circumstances, occur in entirely different forms, and with quite different properties. It was thus brought into genetic relation with *Bacteria*, *Chalara*, *Saccharomyces*, etc. Both Brefeld and Hansen have carried out numerous investigations with this fungus, and have undertaken culture experiments, which were continued for a long time without producing any other than the ordinary *Oidium* form. Recently, it is true, Brefeld has discovered a formation of conidia resembling chains of *Oidium* cells in several higher fungi, but it has not yet been determined whether this also includes that particular species which we designate *Oidium lactis*.

The transparent, thin-walled hyphæ, often forked and branched, form a thick white felt; in the upper part of the filaments transverse septa are formed close together, after which the single cells, filled with very refractive protoplasm, are detached as conidia (Fig. 42: 3 to 7, 11 to 14, 17 to 19). As a rule, the conidia, in longitudinal section, are rectangular

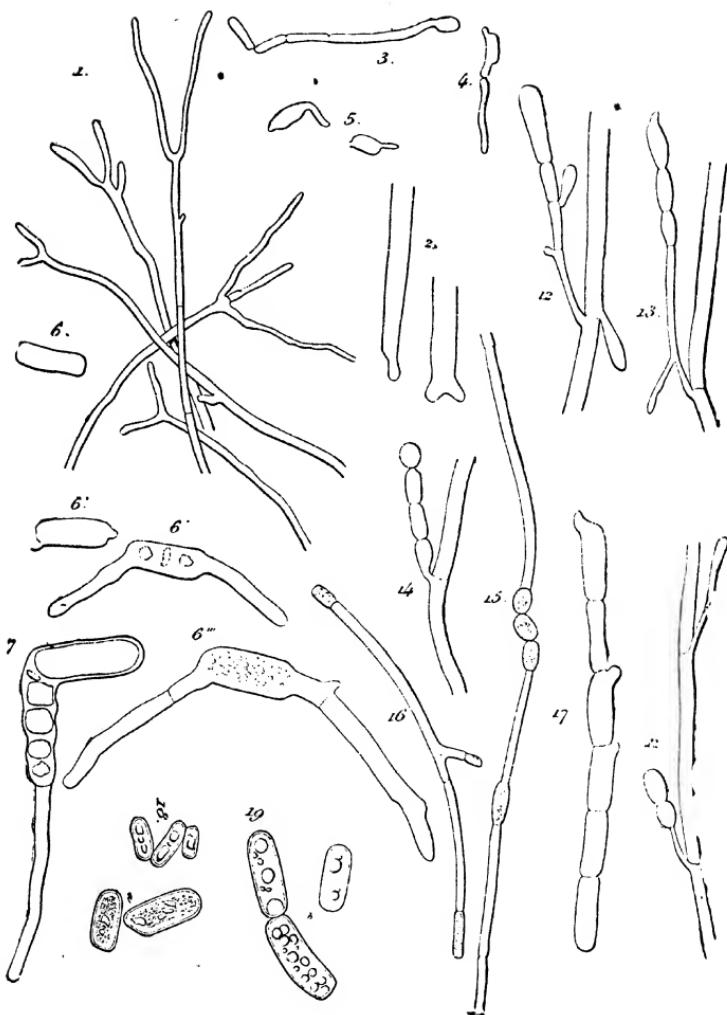


Fig. 42.—*Oidium lactis* (after Hansen).—1, Hypha with forked partitions; 2, two ends of hyphae—one with forked partition, the other with the beginning of a formation of a spherical link; 3-5, germinating conidia; 6-6'', germination of a conidium, sown in hopped beer-wort in Rannie's chamber, and represented at several stages; at each end germ tubes have developed; after nine hours (6'') these have formed transverse septa and the first indications of branchings; 11-14, abnormal forms; 15, 16, hyphae with interstitial cells, filled with plasma; 17, chain of germinating conidia; 18, conidia which have lain for some time in a sugar-solution; the contents show globules of oil; 19, old conidia.

with rounded corners (Fig. 42: 3, 6, 17 to 19); in a growth of this mould spherical, oval, pear-shaped conidia, and others of quite irregular form are, however, almost always present (Fig. 42: 4, 5, 11 to 14). These organs of propagation, the only ones known, send out one or more germ tubes. When the fungus grows on solid substrata, the hyphae unite and form remarkable conical bodies.

Fresenius correctly gave to this species the specific name of *lactis*; for universal experience shows that it has its ordinary habitat in milk, where it can usually be found. It also occurs spontaneously in various other liquids, and among these in the saccharine liquors which are employed in the fermentation industries: in the latter it is able to induce a feeble alcoholic fermentation. Thus, according to Lang and Freudenreich, it produces in milk and grape-sugar solutions, in the course of about ten days, 0.55 per cent., and in five weeks, 1 per cent. by volume of alcohol; smaller proportions of alcohol are produced in cane-sugar and maltose solutions. Its maximum temperature is, according to Hansen, 37.5° C., and its minimum below 0.5° C. Cultures made in lactose nutritive solution develop a powerful odour, resembling that of soft cheese, such as Limburg cheese. *Oidium* is thought by Weigmann, Conn, and others to play some part in the ripening of Camembert cheese. It is believed that *Oidium* is of importance in the ripening of this and other kinds of cheese, because it absorbs the acids produced by lactic acid bacteria, and thus paves the way for peptonising bacteria. Casein, in sterilised milk, is rapidly decomposed by the fungus. According to O. Jensen, an *Oidium* is always present in rancid butter.

The fungus may occur in beer, especially when poor in alcohol. As the amount of alcohol increases, the conditions for its growth become less favourable; still, neither wort nor beer is exposed to the danger of being attacked to any extent by *Oidium*, since it is not able to compete in the struggle for existence with the crowd of organisms which at once appear when fermentable liquids are exposed to the atmospheric germs.

In numerous investigations with top-fermentation yeast, the author has found that it offers a very favourable nutritive

material for this fungus, especially when the yeast is in a quiescent state at the end of the fermentation. Sometimes a microscopic examination has shown an enormous number of conidia. It is not known what influence such a growth exercises on the quality of the yeast and the beer, but without doubt it is advisable to avoid the fungus as much as possible. It forms vigorous growths on pressed yeast also, which have a deleterious action on the quality of the yeast. A large number of species and varieties find shelter under the name *O. lactis*. Weigmann has identified several, and Grimm also isolated a number of forms from sour milk, cheese, etc., which differ clearly from each other both in regard to their characteristic growths on gelatine, and especially on potatoes, and also in regard to their peptonising action on the substratum.

#### 7. Fusarium.

The red colour occasionally occurring on malt grains is due to various fungi, among which is a *Fusarium* described by Matthews and Klein. The mould formation begins on the germinating part of the grains, and thence spreads over its surface. The filaments of the mycelium, which show globular swellings, are connected by numerous bridgings. The red colouring matter is present in the contents of the filaments. On a moist medium the membranes gradually swell, forming a slimy envelope, which is coloured violet by iodine. The oval conidia germinate either directly, or previously grow into sickle-shaped multiple cells. Germinating filaments issue from the points of the latter, and by slow degrees the cells swell up. Both the mycelium and the sickle-shaped conidia are able to produce thick-walled spores like gemmæ. The fungus does not appear capable of hindering the growth of sound malt grains, even if its mycelium spreads freely over their surface. Generally speaking, it only attacks diseased grains.

#### 8. Chalara.

*Chalara mycoderma* (Fig. 43) is described in Pasteur's *Etudes sur la bière* as one of the organisms commonly occurring

on grapes. The mycelium forms a film on liquids, and consists of branched, greyish filaments, which at different points develop conidia of unequal form and size filled with glistening and highly refractive protoplasm. The mycelium frequently divides up into separate *Oidium*-like cells. Cienkowsk first gave a detailed description of *Chalara* in his memoir on the

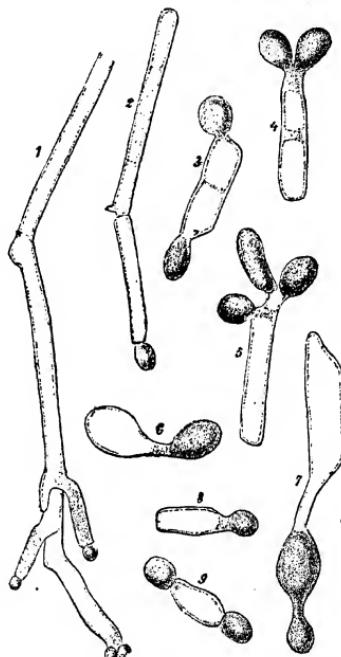


Fig. 43.—*Chalara inycoiderma* (after Hansen).—1, A branched hypha, the terminal limb of which is throwing off conidia; 2, a hypha, at the upper cell of which a sterigma, which has thrown off conidia; 3-9, various forms of hyphal links, which are separating conidia.

film-forming fungi. Hansen found that this mould develops both in ordinary wort and lager beer, as well as in the diluted liquors.

#### 9. *Dematium pullulans*.

A mould about which a great deal has been written in the literature of our subject is *Dematium pullulans* (Fig. 44), which was first described by de Bary, and more minutely by Loew.

It frequently occurs on fruits, especially grapes, and has a branched mycelium from which buds are thrown out; these have a striking resemblance to ordinary yeast cells (4), and are able either to propagate through many generations, by yeast-like budding, or to produce germinating threads giving

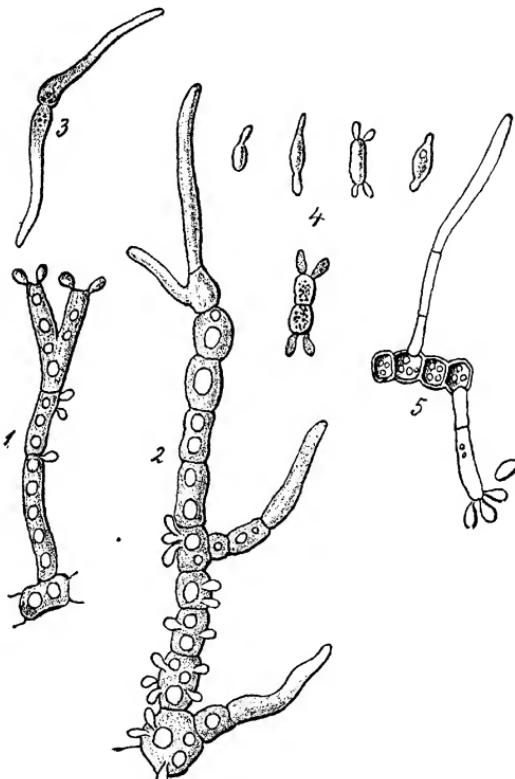


Fig. 44.—*Dematioidium pullulans* (after Loew).—1, 2, Full-grown mycelial threads with yeast-like cells; 3, cells of the latter developing to mycelial threads; 4, cells with yeast-like buds; 5, appearance of yeast-like cells on the germ tubes of the cells, with brown covering.

rise to a mycelium (3). Skerst states that the mycelium develops more particularly at low temperatures, whereas the separate cells form at temperatures of 19°-32° C. In a strongly concentrated grape-sugar solution the fungus chiefly

develops mycelium. When this has attained a certain age, it forms numerous, closely contiguous, transverse septa, and gradually turns brown or olive green (5); this forms the resting stage of the plant. In Hansen's air analyses, *Dematium* was frequently found from spring until late autumn in wort to which air had access. He observed that when the mould was sown in a saccharine liquid it at first developed only mycelial threads; after some time, however, yeast-like cells separated, without inducing alcoholic fermentation.

P. Lindner states that one *Dematium* species produces a ropiness when cultivated in wort, owing to the formation of slime from the cell membrane. *Dematium* species are also found in milk and dairy products. A great development of *Dematium* occurs in the sap which oozes from the cut stem of the vine, and, according to Wortmann, this is the main reason

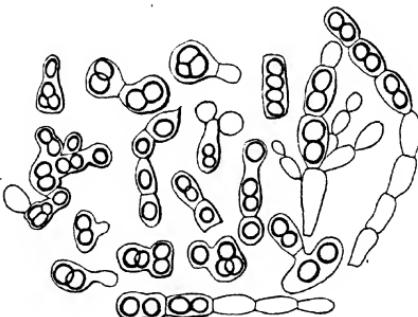


Fig. 45.—*Dematium* species (Jürgensen).—Spore-formation in mycelial threads.

why the sap is gradually converted into a slimy, gelatinous mass. Wine must may also turn slimy, for the same reason, if it is allowed to ferment too slowly. In isolated cases Wortmann observed that *Dematium* exercises a destructive influence on grapes. The author observed in 1895 endogenous spore-formation in *Dematium*-like moulds occurring on dried grapes, but the organism showed no development of the resting cells described above (Fig. 45). The spores developed nothing but a yeast growth in saccharine liquids. The yeast thus developed was capable of spore-formation, and is, therefore, a true *Saccharomyces*.

10. *Cladosporium herbarum* (Fig. 46).

This mould occurs along with others in fermentable liquids, in the fermenting rooms, and also on hops, malt, etc. It sometimes occurs in very large quantities in the fermenting rooms. The author found, in one case, that the ceiling and a portion of the walls in a bottom-fermentation room were thickly covered with small black patches; these consisted of *Cladosporium*, the conidia of which were consequently always present in the yeast. The plant consists of a yellowish-brown mycelium with short, straight filaments, stiff and brittle; those growing erect can produce at their upper extremities conidia of very varying form—spherical, oval, or cylindrical, straight or curved. In contrast to *Penicillium*, where the new conidia are formed basi-petally (*i.e.*, below those which have already been cut off), they rise, in the case of *Cladosporium*, either apical or lateral, from a kind of budding of the mother-conidium, the development being thus basifugal. The name *Cladosporium herbarum* doubtless includes several closely related species. According to Janszewski's researches, the same species can appear in different forms and with a varying size of cell. He showed that the commonly occurring species represents a stage in the development of an Ascomycete (*Mycosphaerella*), the perithecia of which bear some resemblance to those of *Aspergillus*.

Wortmann includes *Cladosporium* amongst those fungi, the mycelium of which, growing through the corks of wine bottles, give rise to the corked flavour of wine. These and other species of fungi occur during the ripening of cheese which,

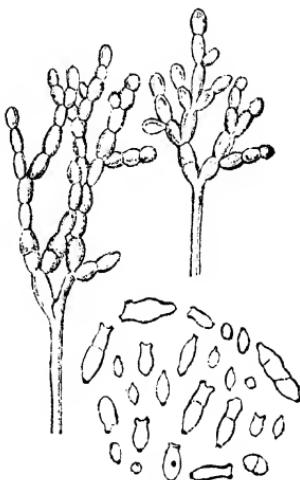


Fig. 46.—*Cladosporium herbarum*.—Conidia-forming hyphæ (Loew) and Conidia (Holm).

through their development, acquires a dark brown or black colour. Fungi belonging to this group play a part in bringing about the decomposition of eggs. Zopf has identified a species which recent investigations have shown to be capable of sending germinal hyphae through the egg shell and membrane, and of gradually decomposing the albumen. O. Jensen has observed that a species of *Cladosporium* promotes the rancidity of butter on account of its power of splitting up fatty substances. Eriksson states that rye is sometimes attacked by *Cladosporium*, and that the mould when consumed in rye bread or in beer may prove pathogenic.

Concerning these, or certain closely related forms, Zopf detailed exact morphological investigations, accompanied by numerous illustrations, in his memoir on *Fumago*, and also in his work on the fungi. This black, soot-like fungus occurs very frequently on plants. Frank correctly says:—"We are still quite in the dark with regard to specific differences, due especially to the frequent polymorphism of these organisms, and to the fact that the different evolution forms are scarcely ever found together."

Among the various fungi occurring on the vine, the two following parasites have obtained an unenviable notoriety, on account of the great damage they cause:—

#### 11. *Oidium (Erysiphe) Tuckeri.*

This fungus, which is also called "the true mildew," forms whitish spots, changing to brown, on the leaves and shoots of the vine. These consist of mycelial filaments, from which elliptical or oblong, colourless conidia separate,  $8\ \mu$  long and  $5\ \mu$  thick. The mycelium spreads over the fruit, which is gradually covered with a delicate growth of a grey colour, while it thrusts through the fruit skin roundish suckers, killing the epidermal cells. When grapes are attacked at an earlier stage, the epidermis is unable to keep up with the growth of the contents; it then gradually splits open like skin affected by scurf, the contents exude, and the grapes either dry up or putrefy. They may impart to wine a very unpleasant smell and taste.

On the full-grown grapes the fungus does not do so much harm, but may still prevent the further maturing of the fruit. The best remedy for this dangerous parasite is sprinkling with finely powdered sulphur, but this only takes effect in sunny weather.

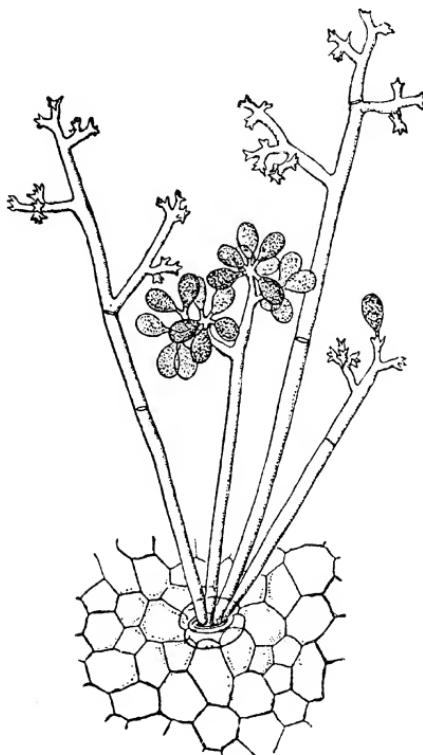


Fig. 47.—*Peronospora viticola* (after Cornu).

## 12. *Peronospora viticola*.

The second vine fungus is "the false mildew," *Peronospora viticola*, which penetrates to the interior of the leaves and fruit, where it spreads and kills the cells. The conidiophores (Fig. 47) burst out from the stomata of the leaves in tufts. The upper part is branched, and both the branches and the

principal axis end in short conical apices. The conidia are oval, 12 to 30  $\mu$  long, and have a smooth, colourless membrane. In the conidia, as a rule, five or six swarming spores are formed, which burst out when the conidia are immersed in water, and penetrate through the epidermis of the leaves and fruit. The growth forms thick, prominent whitish spots on both leaves and fruit. In the interior of the plant, big, globular oospores are formed (30  $\mu$  diameter), which have a brownish membrane, smooth or slightly fluted, and are surrounded by the thin, colourless, or yellowish, oogonium wall. This fungus causes great injury, because the grapes either wither away or putrefy according to the stage at which they are attacked; moreover, it destroys the foliage. The species is indigenous to North America, and was introduced into Europe in the year 1878 along with American vines; it has now spread to all vine-growing countries. Vine growers are endeavouring to suppress this pernicious parasite by the application of copper sulphate and calcium hydrate (Bordeaux mixture), and by similar remedies.

## CHAPTER V.

## YEASTS.

ACCORDING to modern usage the word "yeast" is used to describe those alcohol-forming fungi which are formed, as a rule, by a process of budding, and which under special conditions form spores in the interior of the budding cells. The old name *Saccharomyces* has been retained to describe these fungi, which are of such great technical importance.

It has already been stated that both the bacteria and mould-fungi possess alcohol-forming species, whilst among the moulds certain bud-forming species also occur.

Mycelium formation has been shown to exist in not a few *Saccharomyces*, and since an endogenous spore-formation also occurs in certain of the moulds, it would appear doubtful whether it is correct to class the yeasts as an independent group of fungi. The direct observation of genetic connection between typical *Dematioid*-like mould-fungi on the one hand, and *Saccharomyces* on the other, makes it difficult to accept the earlier view. This observation at all events proves that species exist which cannot be classed in an independent group. Doubtless future investigations will bring to light further instances of species which represent stages in the development of higher fungi.

The genera *Mycoderma* and *Torula*, which include no members exhibiting endogenous spore-formation, but include a number of species known only in the budding stage, will be dealt with in an appendix to this section.

## The Nutrition of Yeasts.

Some account of the nutrition of fungi in general has already been given in the chapter on moulds. In the following

paragraphs a review is given of the special features which yeasts present in this respect.

The inorganic substances—phosphorus, potassium, magnesium, and sulphur—have been enumerated by A. Mayer as indispensable for the nutrition of yeasts. His statement is based on the results of his analyses of yeast (not pure cultures) and of his nutritive experiments. As is well known, yeast contains considerable quantities of phosphoric acid. In Munich brewery yeast, the proportion of phosphoric acid represents about  $3\frac{1}{2}$  per cent. of the dry residue. If malt-wort or other nutrient medium contains too small a quantity of phosphoric acid, this defect may be remedied by adding potassium phosphate. Potassium is also an essential food element for yeast. It is readily absorbed, both in the form of phosphate and sulphate. Magnesium is an element of equal importance. Sulphur can always be detected in yeast, and must, therefore, be regarded as essential to its metabolism. Calcium, on the contrary, does not appear to be necessary for the propagation of yeast. This element plays an important part, however, in the fermentation process, for it has been shown by Seyffert that brewery yeast quickly degenerates in a wort poor in lime. According to recent work carried out by Delbrück, Lange, Henneberg, Hayduck, Seyffert, and others, calcium carbonate is of importance by rendering certain poisonous substances innocuous which are present in the raw materials, and are believed to be of an albuminoid character (see Chap. i.). In the preparation of artificial nutrient solutions for yeast, these substances should be added in the form of salts, the total quantity not exceeding about 1 gramme per litre.\*

\* Ad. Mayer made use of—

Acid potassium phosphate,  $\text{KH}_2\text{PO}_4$ ,

Crystallised magnesium sulphate,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,

Tribasic calcium phosphate,  $\text{Ca}_3\text{P}_2\text{O}_9$ ,

beginning with greatly reduced quantities.

Laurent made use of a solution containing per litre—

0.75 gramme potassium phosphate.

5.0 grammes ammonium phosphate or sulphate.

0.1 gramme magnesium sulphate.

1.0     ,,     tartaric acid.

Molisch found that small quantities of iron exert a favourable influence on the propagation of pressed yeast. Kosowicz showed, moreover, that in this respect ferrous sulphate has a much more favourable effect than ferric chloride.

With regard to the importance of carbon compounds for the nutrition of yeast, Laurent, in particular, proved that yeast can assimilate, in addition to sugar, large quantities of such compounds as lactic acid, glycerine, dextrin, tartaric acid, etc., but not oxalic acid and its potassium and ammonium salts. In practice, sugars play the chief part in nutrition, but the species of yeast differ amongst themselves in this respect, as well as in their power of fermenting these carbohydrates. Thus, according to Beijerinck, *Schizosaccharomyces octosporus* can readily assimilate maltose, glucose, and laevulose, but not saccharose and lactose.

Glycogen constitutes an essential part of the cell contents at a certain stage of its life. This is specially the case when the liquid is rich in carbohydrates, which are stored up as reserve material in this form. Glycogen was discovered by Errera in yeast, and has been more closely studied by other investigators. Laurent proved that yeast can store up very considerable quantities. It appears in the cell as minute semi-fluid drops, with no definite form, and gives a reddish-violet coloration with iodine, which disappears on warming. When the food supply shows signs of becoming exhausted, the yeast cell falls back upon its glycogen.

With regard to possible sources of nitrogen for yeast, it may first be noted that amongst inorganic sources the ammonium salts are readily absorbed. It was proved by Pasteur that yeast can grow in a nutritive fluid containing no organic nitrogen, but only nitrogen in the form of ammonium tartrate (100 c.c. water, 10 grammes sugar, 0.1 grammes ammonium tartrate, and the ash from 1 grammes of yeast). Subsequently Willdiers

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Hansen's artificial culture fluid contained—

88.5 grammes distilled water.  
 0.2 grammes magnesium sulphate.  
 0.3   ,    monopotassium phosphate.  
 1.0   ,    peptone (Witte).  
 10.0 grammes saccharose.

proved by exhaustive experiments that it is not sufficient to supply nitrogen in inorganic compounds. Kossowicz reached the same conclusion, and showed that by sowing a single cell in a saccharine fluid containing mineral matter no development took place, whilst with a greater infection of cells development may proceed.

It has been definitely proved in the case of the *Mycoderma* species that their demand for nitrogen can be fully satisfied by ammonia in the form of inorganic compounds.

Organic nitrogenous compounds occur in considerable quantities in most of the liquids fermented in practice. Thus the cereals which are utilised in breweries and distilleries contain a series of proteins which possess a nutrient value for the yeast cell. Rye is specially rich in these substances, and for this reason an admixture of this cereal is always used in the manufacture of yeast. Peptones and amides are formed during the mashing process, both of which appear to be absorbed by yeast with special facility. Asparagin, which is present in considerable quantities in sprouted corn, malt, and potatoes, plays an important part as a source of nitrogen; the yeast converting it into protein. Cider musts are notably poor in nitrogenous food for yeasts. Müller-Thurgau, therefore, recommends the addition of ammonium chloride (about 20 grammes per hectolitre) or of ammonium tartrate.

A large number of analyses have been made to determine the albuminoid contents of yeast. Wijsmann found that the proportion varies greatly, even at different stages of fermentation. At first the quantity of nitrogen rises rapidly, but afterwards it gradually diminishes. Thus the nitrogen may rise from about 7 to 10 per cent., calculated on the dry substance, during the first hour. Amongst the albuminoids, special reference must be made to the nucleins, substances which owe their name to the fact that they are the principal constituents of the nucleus of the yeast cell. They were shown to be present in considerable quantities in Kossel's extensive researches on pressed yeast, and others have since demonstrated their presence in yeast. They are very complex substances, and appear to play an essential part in the development of the yeast cell (division of the nucleus). Their occur-

rence in the cell may be demonstrated by micro-chemical methods—*e.g.*, by the action of pepsin (dissolved in 0.2 per cent. hydrochloric acid)—which, according to Zacharias, attacks the other albuminoids, but not the nucleins; they are, however, dissolved by weak alkalies.

The great importance of yeasts, both from the scientific and practical standpoint, is due to their power of forming alcohol from the sugars. From time to time numerous attempts have been made to explain the actual processes which go on during the course of fermentation. It is only quite recently that a starting point has been found for the investigation, which has made it possible to subject this physiological activity of the yeast cell to experimental treatment. The labours of previous workers in this field have, however, produced results of the highest scientific and practical value, and not a few of the investigations in this and adjacent fields of research have laid the basis upon which modern views are built. Our statement must, therefore, be based on a *résumé* of the entire development which has led, during the course of years, to the various theories of fermentation.

### Theories of Fermentation.

It was long ago observed that when a sugar solution or fruit juice is exposed to the air, fermentation phenomena occur after a certain lapse of time. The liquid becomes turbid, an evolution of gas takes place, a precipitate is formed, and the surface is covered with a layer of yeast. The liquid gradually loses its sweet taste, clarifies at the same time, and then proves to contain a new substance with a stimulating action.

What exactly the process might be was the object of many speculations in olden times, which were not based upon any true investigation of the processes. We will put all these speculations on one side, and start with the end of the eighteenth century, the time of the renowned Lavoisier, the founder of modern chemistry, who gave the first explanation of the phenomena based on facts, the first link of a theory of fermentation. He proved that simultaneously with the dis-

appearance of sugar, spirit of wine, carbon dioxide, and acetic acid were formed. He explained the process as the splitting up of an oxide into substances both poorer and richer in oxygen. As the yeast played no part in determining the quantitative ratio of these he did not concern himself further with it.

At the beginning of the nineteenth century Gay-Lussac published the well-known equation of fermentation, which still holds good, according to which a molecule of grape sugar \* was decomposed into two molecules of carbon dioxide and two molecules of alcohol. The basis was thus given for a definition of fermentation—viz., a breaking down of complex bodies into bodies of simpler construction.

The question now arose in what way this transformation was brought about; what was the true cause of the decomposition of the liquid?

In the literature of the seventeenth, and still more in that of the eighteenth century, allusions are made to a "ferment" (Willis, Stahl) which was declared to be "a body existing in a state of internal motion which transfers its motion to other bodies present in the liquid, whereby the coupling of the compounds present is torn apart. The fragmentary particles are, however, through constant friction, attenuated and transformed into a new and more stable compound." These indications, however, remained unheeded. In 1810, Gay-Lussac, encouraged by the brilliant chemical discoveries of Lavoisier, undertook experiments to elucidate the process of fermentation, starting from Appert's method of preparation, which consisted essentially of preventing organic matter from undergoing fermentation by boiling it, and immediately afterwards sealing it tightly in vessels so that no air could penetrate to it. This process was, however, no new one, for as early as 1782 the Swedish scientist, Scheele, proved that acetic acid can be preserved unaltered after subjection to heat.

Gay-Lussac examined the air contained in such hermetically sealed vessels, and found that it contained no oxygen. In his *Zeitalter des Sauerstoffs*, this observation led to the view that oxygen itself was the true cause of the process of

\* Not cane sugar, as Gay-Lussac believed.

fermentation, a view confirmed by the practical experience that sulphurous acid could be used for fuming out casks to arrest the fermentation of must, because the conversion of sulphurous acid into sulphuric acid brought about the removal of oxygen from the air of the casks.

The importance of yeast for the fermentation process was quite overlooked. It was regarded as a precipitation from the liquid of no further importance for the comprehension of the process. The first indication of the true relationship had, however, been discovered at a much earlier period.

About the year 1600 two Dutchmen, Hans and Zacharias Janssen, invented the microscope, and in the latter half of the seventeenth century another Dutchman, Leeuwenhoek, issued his renowned letters on the investigation of different substances undertaken with the help of this instrument for the Royal Society. In 1680, in the course of one of his letters, a description and drawing of beer yeast appeared for the first time, and later in the same year one of wine yeast. Shortly after the first clear sketches of bacteria appeared. He held the view that the globular yeasts were derived from the flour of cereals used in brewing, and he compared them with starch granules. He had, however, no glimmering of the importance of yeast for fermentation.

The observations of the learned Dutchman regarding the microscopical "animals," for so he named the bacteria, originated the great discussion which has extended into our time, involving researches and explanations of the important question as to whether these organisms can be derived from inorganic and dead matter, or whether they are derived from external fermenting and living matter. The whole of the following development is based upon the investigation of this question, which naturally had its influence in the domain of fermentation.

After the fairy tales of earlier times had been disproved, Needham came forward in 1745 with definite experiments designed to show that the lowest microscopical forms of life, the "infusoria," were created in the following way:—He exposed decoctions of meat and other organic substances to such a high temperature that, according to his views, all forms of living matter must be killed, and the vessels were

then hermetically sealed. When he opened them later he found living "infusoria" in the material, and he naturally argued that they were spontaneously generated, and that the substances liberated during decay had combined again and formed these microscopic forms of life.

Needham's experiments were sharply criticised by Spallanzani in 1765, who proved that if decoctions were maintained for three-quarters of an hour at the boiling point, no living forms were developed until air was admitted. These experiments, incidentally, gave a rational basis to the processes of Scheele and Appert. Needham replied that this result could be explained in a perfectly natural way by assuming that the air present in the vessels was so altered in its character by continued heating that it was no longer able to maintain life. Spallanzani was unable to combat this view experimentally, and so the matter remained undecided, and each view had its supporters. The Needham school was supported by the observation of Gay-Lussac that air in hermetically-sealed vessels contained no oxygen.

No progress was made until the year 1836. From this time on begins a period of rational investigation. Franz Schulze proved for the first time that oxygen does not play the part that had previously been assumed, and his experiments also led to the introduction of the first indications of a biological theory alongside the dominant chemical theory. Schulze vigorously boiled a mixture of water and organic matter in a glass flask, and then allowed air to pass through the flask after bubbling through sulphuric acid. This was carried on daily for a long time. The result showed that the contents of the flask could be preserved unaltered for months together, whereas living forms of matter appeared in the decoction as soon as the vessels were opened and exposed to the access of air.

At the same time Schwann carried out a similar experiment, with the exception that air was passed through a red-hot tube into the decoction. He obtained identical results.

But, although these experiments proved that the air present in the flasks contained oxygen, and that living matter was not produced, they proved unconvincing to the supporters

of spontaneous generation. They fell back on the theory that the powerful treatment the air had undergone had so altered its composition that it was no longer able to produce life.

Schroeder and Dusch took up the subject in 1853-1861, with the object of proving that air containing all its gaseous constituents, unaltered, may be allowed to react on boiled fermentable material without effect. It is unnecessary to expose the air to any vigorous chemical treatment with strong reagents, if it is first separated as far as possible from solid particles. For this purpose they made use of a filter of cotton wool, through which the air was led before it came into contact with the boiled organic mixtures. Boiled meat and meat-broth, as well as malt-wort, were unaltered when filtered air was introduced into the flasks. On the other hand, the experiments did not succeed with milk or the yoke of egg stirred up in water and boiled. A completely decisive proof could not be furnished by Schroeder until, in 1861, he succeeded in sterilising this substance. About this time Pasteur had begun a number of his epoch-making researches, in which the principle of sterilisation was clearly established.

It appeared clear, therefore, to Schroeder that in certain cases before filtration the air must contain something that could bring about fermentation and decay. Whether these are "floating, microscopic, organised germs in the air, or a chemical substance, as yet unknown, which is separated by contact action and fixed on the cotton wool, must remain to be determined." It also appeared to be probable, after correction of the unsuccessful experiment, "that lower infusorial ferments exist, produced and separated either from living plant cells or from living animal tissue, which are capable of exercising certain organic functions and transformations." Mention must also be made of the experiments begun by Hoffmann in 1860 regarding decay and fermentation. He boiled the organic matter in a flask with a long drawn-out neck bent several times at an acute angle. The subsequent inflow of air during cooling deposited dust by gravity so that none could fall into the liquid.\* The result was exactly that obtained by filtration; the liquid remained unaltered. Not-

\* A similar arrangement was made use of by Chevreul.

withstanding all these observations, the school of spontaneous generation maintained their belief, and still numbered many adherents.

In 1857 Pasteur, the distinguished French scientist, entered the field. He submitted the problem to such conclusive experimental treatment, from every side, that his conclusions were generally adopted, and have been held ever since. He proved that the many unsuccessful experiments designed to overthrow the doctrine of spontaneous generation were occasioned by the fact that the organic liquids concerned had not been exposed to a sufficiently high temperature, or heated for a sufficiently long period. Moreover, he showed that in such cases the liquid under treatment was not so greatly altered that it was no longer fit for the development of the germs, as the supporters of spontaneous generation maintained. Thus, if the liquid is boiled in a flask, the neck of which is drawn out into a tube and twice bent (the same idea as that of Hoffmann and Chevreul) so that the liquid remains sterile, and if a small portion of the liquid is then allowed to run into the tube, it soon begins to ferment, owing to the germs deposited in the tube coming in contact with the liquid. The same thing occurred when the air is passed through cotton-wool and a small quantity of the wool is introduced into the sterilised liquid. Pasteur also employed gun-cotton in place of ordinary cotton-wool. Air passed through gun-cotton was sterilised, and the fluid, after boiling sufficiently, remained sterile for an unlimited time. The gun-cotton was afterwards dissolved in alcohol and ether, and it was proved that it contained the same microscopical organisms that develop in liquids undergoing fermentation and putrefaction.

This great work of Pasteur's resulted in the overthrow of all proofs previously adduced on behalf of the school that maintained the spontaneous generation of microscopical life in organic liquids. He established the extremely important result for industry which embodied all the essential principles of the technique of sterilisation. A high stage of development of this technique has since been reached, both in its purely scientific and practical aspects.

Thus was laid the foundation of the belief that fermenta-

tion is brought about by living matter, the vitalistic theory of fermentation, in contrast to the chemical theory which found its best-known advocate in the distinguished Liebig, who built largely on the theories propounded by Willis and Stahl, after Gay-Lussac's idea that oxygen was the direct cause had been given up.

Although Liebig's theory has been abandoned, it is necessary, even in a brief historical description, to touch upon it, because it held the field for a long time, on account of its author's great renown.

Experimental chemistry had won great triumphs in the last twenty years of the seventeenth century. Chemists had succeeded in ascribing extremely complex organic processes, previously ascribed to the mysterious vital energy, to a simple action of chemical affinities. As an obvious consequence, the attempt was made to explain fermentation phenomena in the same way, without the help of living beings. Liebig, however, regarded the yeast which appeared in the fermenting liquid as a substance constantly undergoing decomposition, by which the chemical motion incidental to these processes was transmitted to the sugar, and brought about the decomposition of the latter. It will be seen at once that this theory could not be held when the presence of living and vigorous yeast cells was recognised. Liebig, however, did not regard yeast as a plant; it represented to the chemist a substance without life, and microscopical investigation, according to Liebig, could contribute nothing of importance to the understanding of the process.

We shall now proceed to discuss how the knowledge of yeast developed and led to the vitalistic theory which prevailed for such a long time.

The Austrian Plenciz declared, as early as 1762, that decay only takes place in a body when "germs of a wormy character develop and begin to multiply." Probably we have here the first definite announcement regarding the cause of such decompositions.

A long time elapsed before Leeuwenhoek's observations on yeast cells, in 1680, carried us a step further. As far as we can judge from the known literature, it is believed that the

Austrian Erxleben in 1818 was the first who definitely expressed the thought that fermentation "appears in no way to be a simple chemical operation, but rather is in part a process of growth, and should be regarded as the link in the long chain of nature which combines those actions that we describe as chemical processes with those of vegetative growth." But this must be regarded only as a hypothesis without further foundation.

Twenty years later, and almost simultaneously, three scientists expressed clear and definite views based on direct experiments regarding the dependence of alcoholic fermentation upon yeast cells.

It may be of interest to see how they arrived at the same result in three different ways.

Cagniard-Latour was the first to publish his work on yeast, in 1835-37. In his studies of beer and wine fermentation, both in practice and on the small scale, he observed that the yeast globules rise to the surface of the beer-wort on account of the entangled gas which they produce. They possess the power, by budding or by elongating their own tissues, of multiplying, and in this way producing manifold globules, which separate from each other when fully grown. He thus confirmed his view that yeast cells are organic, and belong to the vegetable kingdom. During propagation they are nourished by the beer-wort, and when the fermentation has come to an end the liquid contains many times the quantity of yeast that was added to it, whereas the earlier view was that the substantial precipitate consisted mainly of secretions. He also found that yeast will not propagate in pure sugar solutions.

His researches enabled him to conclude that in all probability it is the yeast cells that destroy the stability of the components of sugar, and bring about its decomposition into alcohol and carbon dioxide; that fermentation, in fact, is a result of vegetable activity.

The same observation regarding the vegetable character of yeast was made simultaneously, or a short time after, and quite independently by Theodor Schwann. It has already been mentioned that he made important contributions to the discussion of the generation of living matter, and it was these

investigations that brought about his exact study of yeast under the microscope in 1837-39. Schwann arrived at the result that it is not atmospheric oxygen, but a substance conveyed in air, and destroyed by heat, which brings about fermentation. To determine whether this substance is of animal or vegetable character, he enquired whether the substance is destroyed by those poisons that are capable of killing infusoria, or by those that kill moulds. The latter proved to be the case, for a solution of potassium arsenite arrested the fermentation of wine; therefore, he argued, the substance must be of a vegetable character.

Under the microscope the yeast resolved itself into the "recognised granules which constitute the ferment." Then he observed how they form continuous rows, with other rows placed diagonally. He also observed that small granules appeared on the sides of the cells, which form the starting point for new rows, and usually on the last granule of a row appeared a tiny and sometimes elongated body. It will be seen that this constitutes an exact description of a budding colony of yeast resulting from direct observation under the microscope. Schwann observed that the similarity between this picture and that of many other kinds of fungi was considerable, and this strengthened his belief that yeast is a plant. At his instigation Meyen examined "this substance," and gave the plant the name it has since retained of *Saccharomyces* (sugar-fungus).

Schwann also demonstrated that the feeble evolution of gas in grape juice may be regarded as a sign of fermentation; immediately afterwards the first individuals of the sugar-fungus made their appearance; these plants grew and multiplied throughout the period of fermentation. As it had also been shown that fermentation ceased through every treatment which brought about the destruction of the fungus (boiling, addition of potassium arsenite, etc.), the connection between fermentation and the sugar-fungus cannot be denied, and "it is extremely probable that the latter brings about the phenomena of fermentation through its growth." He declared that fermentation was carried out in such a way that "the sugar-fungus absorbs sugar and a nitrogenous body necessary for its

nutrition and its growth, whereby those elements which are not taken up by the vegetable body, are principally combined to form alcohol (probably along with many other substances)."

F. T. Kützing was the third who dealt with this important problem at the same time (1834-37). Within the scope of his elaborate investigations concerning the lowest microscopical plants he included the yeasts and other micro-organisms that usually occur in brewery wort and distillery mash, and published good drawings of these growths. It is of particular interest that Kützing was the first to investigate the mother of vinegar, the slimy skin which forms on the surface of a liquid that is undergoing acetic fermentation. He examined this film from its earliest stage, and found that it consisted of very small plants, which gradually increase in length. He realised the extraordinary importance which the study of the lowest forms of life would have for organic chemistry, and for the whole field of natural science. Chemistry must rule out yeast from amongst its chemical compounds, as it proves to be an organism, and he regarded it as certain that "the whole process of the spirituous fermentation is dependent on the formation of yeast, and that of acid fermentation on the formation of mother of vinegar"; "fermentation is synonymous with the vital process." Thus he supplied a clear and definite form for the vitalistic theory of fermentation in opposition to Gay-Lussac's oxygen hypothesis, and to Liebig's theory of the breaking down of yeast cells as the cause of fermentation.

Mitscherlich's work is also of a fundamental character. In 1841 he described the yeast as consisting of round and oval globules, and he solved the question of their importance for fermentation through the following beautiful experiment:—A little yeast is placed in a glass tube, closed at the lower end with a sheet of paper, and this is placed in a sugar solution. In the course of several days it will be seen that fermentation has actually taken place in the tube, owing to the sugar solution having diffused through the paper. Alcohol gradually diffuses out throughout the liquid, which becomes saturated with carbon dioxide, but the greater quantity of carbon

dioxide is evolved from the tube. It is only after some time, when the paper softens and allows the yeast globules to pass through, that the fermentation process begins to take place on the surface of the paper. He concludes that "fermentation only takes place at the surface of the globules." He also published beautiful drawings of yeast, showing their methods of growth and propagation, and described the contents of the cell after staining with iodine.

All these observations did not suffice to establish the new theory. The great authority of the chemist, which still prevailed, required an equal authority in the region of biology to take up every point of the discussion, and by convincing experiments along the whole line, to compel attention; lacking such an authority, the earlier disputants were unable to win the victory.

This great work was carried out by Pasteur with the same conclusive results as in the case of generation. The investigations begun by Pasteur did not consist, like those of the earlier experimenters, of short, isolated pieces of work, but ranged over a series of years from 1857 onwards, and were published in a number of memoirs. In this short review it is impossible to do more than indicate a few isolated and especially important experiments taken from the series, which ranges over the whole field of fermentation.

At an early stage he made the important observation that the amount of sugar dissolved during fermentation is greater than that corresponding to the carbon dioxide and alcohol produced. The remainder of the sugar that disappears is utilised by the yeast during fermentation, partly for its propagation—a circumstance which cannot be reconciled with Liebig's view, who demanded as a condition of fermentation that yeast should be in a state of decomposition. Shortly after, Pasteur proved that during fermentation yeast not only produces alcohol and carbon dioxide, but simultaneously succinic acid and glycerine, the latter derived from a further part of the disappearing sugar. He also showed that by the addition of ammonium tartrate to the fermenting liquid yeast can be brought to more rapid development, and the liquid can be more highly fermented than usual, proving that this salt

must be a food-stuff for yeast. In general, he proved that no decay of yeast takes place during fermentation, and that the presence of assimilable albuminoids in the liquor is unnecessary, for by sowing a minute portion of yeast in a liquid which only contains sugar, ammonium tartrate and a few salts, fermentation can be brought about with development of young cells capable of propagation.

At the same time, he showed that the reason why many of the early experiments, which should have refuted the older theories, did not succeed was due to the fact that it was impossible at the time to secure absolute sterilisation of the liquids.

He then produced further proofs that the acetic acid fermentation, already recognised by Kützing as due to physiological activity, must certainly be regarded as having this character.

One further observation must be mentioned on account of its wide-reaching importance. He proved that calcium lactate can undergo fermentation resulting in the formation of butyric acid, and that the active organism can exist without access of air. He gradually extended his observations in this entirely new field (*anaërobiosis* = life without air), and definitely distinguished between aërobic and anaërobic life. It was this remarkable discovery, which at a later stage included the alcoholic yeasts, that led the distinguished scientist to a solution of the problem under what conditions yeast cells can decompose sugar.

In 1876, in his *Études sur la bière*, he formulated his celebrated theory of fermentation, based upon a series of actual experiments, details of which cannot be given here, a theory which has served both as the basis and the starting point by which progress has been made throughout a long series of years, and one which will always retain its importance. It starts essentially from the thought that living yeast cells under certain conditions are obliged to live apart from air, and that they then react as excitors of fermentation. Fermentation is, therefore, bound up with the life of yeast cells; it is life without air. As yeast under these conditions is obliged to obtain its necessary demands of oxygen from sugar in order

that it may continue to develop as a living organism, it splits up the sugar, and the residue of the oxygen, as well as the carbon, constitute new compounds—viz., the fermentation products, alcohol, carbon dioxide, etc. At the same time, Pasteur emphasised the idea that for each kind of fermentation—alcohol, acetic acid, butyric acid, etc.—a specific kind of organism occurs.

It will be seen that Pasteur's theory consists both of a biological and a chemical portion. The yeast cells fulfil their normal existence with generous access of atmospheric oxygen, and under these conditions develop, according to his view, most vigorously, and prepare themselves in the best possible way to continue their existence without air, and this is the necessary condition for their existence as alcohol formers—i.e., decomposers of sugar. The first statement, which clearly explained an important biological problem, still holds good; the second, which endeavoured to supply an answer to the requirements of the chemical process, can no longer be accepted.

That Pasteur did not apply his definition in the narrowest sense of the word is shown by the fact that he himself emphasised the fact that yeast can exercise fermentative power in presence of a limited supply of air as well as in its absence. This was established under certain conditions for low fermentation beer-yeasts by Pedersen in 1878, and Hansen in 1879. They arrived at the result that the quantity of dry substance in beer-wort which a given quantity of yeast can convert into alcohol, carbon dioxide, etc., is smaller when the liquid is aerated during fermentation than when it is not. A similar result was obtained by Eduard Buchner in 1885 in his experiments on bacteria.

Hansen so arranged his experiments that the cells during aeration were in constant motion, carried hither and thither by the vigorous blast of air. As they, nevertheless, continued to give a distinct alcoholic fermentation, there can be no doubt that this is not determined by life without air.

Nägeli, in 1879, in his *Theory of Fermentation*, proved that access of oxygen is always favourable to alcoholic fermentation in a sugar solution, if no nutritive material is present, and

consequently the quantity of yeast is only slightly increased. Nägeli says (p. 26), "The theory of Pasteur, that fermentation results from a lack of oxygen, forcing the yeast cells to secure their requirements of oxygen from the fermenting material, is opposed to all the facts brought to bear upon this subject."

This view is shared by A. J. Brown. He arranged a set of experiments in which fermentation proceeded in presence of full access of oxygen, and a parallel set in which oxygen was excluded. In both series the same number of yeast cells were used, and they were kept under such conditions that it was impossible for them to multiply. Otherwise every condition was the same. It proved, contrary to Pasteur's theory, that the cells in the first case developed a higher fermentative activity than when oxygen was excluded.

Hueppe and his pupils have also rejected Pasteur's theory of fermentation, and have brought forward examples of ferment organisms "which are able to bring about the specific fermentation much better, on the whole, in presence of atmospheric oxygen."

Similar experiments were undertaken by H. Buchner and Rapp, with the object of ascertaining by exact quantitative methods to what extent free access of air brings about the replacement and suppression of the fermentative power of yeast cells by their oxidising function. With this object in view, they prepared pure surface cultures of yeast with the greatest possible access of air, and carried out parallel experiments with limited access. The first lot of cultures were grown in large cylindrical vessels, the inner wall of which was covered with a thin lining of wort-gelatine containing 10 per cent. of grape sugar. This was infected with a coating of pure yeast, and in each experiment a current of air was passed through the vessel for five days. The carbon dioxide was absorbed in caustic potash, and after each experiment the amount of alcohol and the ratio between the yeast and the fermented sugar were determined. Parallel experiments were carried out, in which the same quantities of beer-wort and grape sugar were allowed to ferment in Erlenmeyer flasks.

As a consequence of the rapid and abundant growth of

yeast on the surface of the gelatine, the fermentation on gelatine ceased much more quickly than in wort, where the yeast collected on the bottom of the flask. It was further proved that considerably more carbon dioxide was formed in the surface cultures than in the parallel experiments with wort. This carbon dioxide must be due to the respiration of the yeast. Nevertheless, only about one-seventh of the sugar was decomposed by oxidation, whilst more than six-sevenths were fermented. Although the yeast had been submitted, according to Pasteur's view, to the most favourable conditions for life without bringing about fermentation, this did not prove to be the case. As is now universally known, a free supply of oxygen exercises a favourable influence on the propagation of cells, but these experiments served to establish the fact that oxygen has scarcely any influence on the process of fermentation, and that the absence of oxygen must not be regarded as being of immense moment for the fermentative activity, for even in presence of a full quantity of oxygen the fermentative power of yeast still exceeds the respiratory power.

From Nägeli's many-sided work on the lower organisms, we can only refer, in connection with the preceding, to his "molecular-physical" theory of fermentation, which may be regarded as a modification of Liebig's theory. Whilst Pasteur regarded fermentation as the result of an activity taking place in the cell, Nägeli defined fermentation as a transference of states of motion of the molecules, groups of atoms or atoms of different compounds, constituting living protoplasm (which otherwise undergo no change) to the fermenting material, whereby the stability in these molecules is destroyed and disruption is brought about. During fermentation the vibrations of the molecules of protoplasm are transferred to the fermenting material. The cause of fermentation is to be sought in the living protoplasm in the interior of the cells, but its activity extends for some distance outside the cells. The decomposition of sugar takes place to a slight extent inside the yeast cells, but principally outside them. This theory is opposed to that of Pasteur, and is related to that of Stahl and Liebig.

We now return to the epoch-making researches of Pasteur. He proved in the clearest and most unmistakable manner in his *Études sur la bière* what power is possessed by microscopic life, and he strongly emphasised the fact that bacteria may have a far-reaching influence on the course of alcoholic fermentation and on the character of the beer. The budding organisms were dealt with in a similar way. He indicated that certain fungi of this group, which are not described in detail, may react in different ways on the products of fermentation, as Bail had previously experienced. Pasteur's communications, however, only traversed the nebulous views of his predecessors, and his assumptions led to two opposing lines of thought. This is seen, for instance, in his observations on the so-called cheesy and aërobiotic yeast. It is possible that we have to do in this case with independent and peculiar types of yeast, but it is also possible that we are dealing with forms which are brought about by a particular treatment of the usual brewery yeast. It should not, however, be overlooked that he himself indicated the direction in which the solution of the question must be sought—viz., that it was at the time impossible to determine whether one or more species was present; an exact method for the pure culture of yeast species had not been discovered. Thus a true orientation in the world of micro-organisms cannot be found in his work. It was impossible at any point in Pasteur's thesis to find characters described for the budding fungi that would enable an analysis to be based upon them. He believed that all budding fungi may to some extent possess the power of bringing about alcoholic fermentation like the *Saccharomyces*. It is never possible to tell whether he is referring to true *Saccharomyces* or to other budding fungi. Pasteur did not differentiate between the several kinds of budding fungi (*Saccharomyces*, *Torula*, *Dematiuim*, etc.).

Pasteur took the standpoint that every individual fermentation, lactic, butyric, acetic acid, etc., is produced by a particular exciter of fermentation. It was only when the technique of pure cultivation had been further developed that an explanation of the true connection was possible—viz., that *each one* of these processes must be carried out by different

kinds of organisms. This was proved by E. C. Hansen in 1878 for acetic acid bacteria, by Miquel for uric acid bacteria, and by Hansen in 1883 for the alcohol yeasts.

The chief reason why the reform in brewing technique could not be carried out was that the existing scientific standpoint made it impossible to clearly define the relations existing between the different fungi concerned in alcoholic fermentation. Pasteur was, therefore, unable to escape from the indistinct assumptions and the contradictory views of his predecessors. In a review given in his book (pp. 4-7) regarding the micro-organisms that bring about diseases in beer, he speaks only of bacteria, and this belief is reiterated by Duclaux in 1883, and by other French, German, and English workers. As a result of his studies, Pasteur recommended brewers to undertake the purification of their yeasts, to rid them of bacteria by cultivating them in a sugar solution with tartaric acid or in wort with a little carbolic acid.

In contrast to all this, in 1883 Hansen published his doctrine that some of the most dangerous and most commonly occurring diseases in low-fermentation beer are not produced by bacteria, but by certain species of *Saccharomyces*, and that the names *S. cerevisiae*, *S. Pastorianus*, and *S. ellipsoideus*, suggested by Reess, do not indicate one, but several different species and races. Hansen proved that species which had been incorrectly grouped under the systematic name *S. cerevisiae* yield different products in the brewery. From this standpoint he elaborated his system, utilising a stock yeast derived from a single species. After some opposition, this system was adopted in all brewing countries, and introduced into the industry. Hansen's experimental demonstration showed that Pasteur's process for purifying yeast by means of tartaric acid furthers the development of disease yeast to such an extent that they are capable of completely suppressing the true culture yeasts. Pasteur greeted Hansen's system as an advance, and wrote, "Hansen was the first to realise that beer yeast should be pure, and that not only in regard to microbes and disease ferments in the narrower sense, but also that it should be free from cells of wild yeast."

The main problem regarding the actual cause of the decom-

position of the sugar molecule and the special conditions under which it took place still awaited solution.

Meanwhile, in the last decade of the nineteenth century, new views regarding the fermentative forces were gaining ground, when it proved possible to separate the exciter of fermentation in certain cases (diastase from malt, pepsin from gastric juice). The characteristic effect of these ferments was that minute quantities were able to split up large amounts of the given material, and that they completely lost this power when subjected to heat. The name enzyme was applied to the substances isolated from the living cells of the barley corn, the mucous membrane of the stomach, etc., and gradually a large number of these ferments were distinguished, amongst them some of great technical importance.

The thought naturally suggests itself that it must be possible to find such an enzyme amongst the many elements of which the living yeast cell is constituted which would be capable of splitting up sugar. As early as 1858 we find a suggestion of this kind put forward by Traube that "the chemical processes going on in living organisms originate mainly in the circumstance that protein substances are liable to undergo decomposition in the presence of water, and that under the peculiar conditions actually obtaining they are also apt to give rise to peculiar ferments." A direct outcome of this view was Miquel's discovery in 1890 that the bacterium causing the ammoniacal fermentation of urine contained an enzyme which can bring about this fermentation on its own account.

In 1894 Emil Fischer, by purely chemical research, resulting in his celebrated work on the synthesis of the sugars, on the use of phenyl-hydrizin, and the osazone-reaction, diverted the current views on fermentation phenomena into new channels. His researches led him to explain the behaviour of the yeast cell towards the particular sugar of the nutritive liquid in the same way as that of the enzymes (invertase, emulsin), so that the chemical activity of the living cell does not differ from the action of chemical ferments. According to Fischer, fermentation of polysaccharides is always preceded by hydrolysis of the sugar. But there exists an exact relation between the

molecular structure of a given sugar and the sugar-inverting enzyme of a yeast cell ; if a sugar comes into contact with the albuminoids of a yeast cell, which play the most important part among the agents utilised by the living cell, the sugar is decomposed only if its configuration, the geometrical structure of its molecules, does not deviate too much from the configuration of the molecules of the albuminoid. Thus, according to Fischer's theory, the function of the living cell depends much more upon its molecular geometry than on the composition of the nutritive material.

Another way in which Fischer, as well as Thierfelder, obtained confirmation of his fermentation theory was by examining the behaviour of Hansen's and other yeast species towards the artificial sugar species, synthetically obtained by Fischer. They found, indeed, that the yeasts are quite fastidious regarding the geometrical configuration of the sugar molecule, whilst they often remain unaffected by other alterations in its composition.

Among the various synthetically-prepared sugars examined by Fischer with regard to their behaviour towards yeasts, *melibiose* is especially mentioned. It is fermented by brewers' common bottom-fermentation yeasts, but not by many brewers' top-fermentation yeasts. In harmony with this, Fischer found that bottom-fermentation yeast contains an enzyme capable of extraction from the dried yeast in aqueous solution, which decomposes melibiose, converting it into glucose and galactose ; but in a corresponding treatment of the brewers' top-fermentation yeasts no decomposition of this sugar could be observed. As brewers' top-fermentation yeast contains invertase, it follows that the ferment which splits up melibiose cannot be identical with invertase.

C. J. Lintner and Fischer showed, by methods devised by the latter, that natural maltose is split up into two molecules of glycose, if acted upon by an aqueous extract of dried yeast, or by cells, the membrane of which has been torn by grinding with powdered glass, and that there is a marked difference between this enzyme and invertase which hydrolyses cane-sugar. The former enzyme is termed yeast-glycase or yeast-maltase. Its optimum temperature is about 40° C., whilst

that of invertase, according to Kjeldahl, is 52°-53° C. In a similar way, a lactose-cleaving enzyme (lactase) and an enzyme resembling invertase were isolated from *Monilia*.

At the same time, Hans and Eduard Buchner were endeavouring to prepare a juice by a treatment of the yeast cells similar to that adopted by Emil Fischer—*i.e.*, by grinding the wall of the cells, hoping to apply it to therapeutic experiments. To preserve the juice, it was mixed with sugar, and E. Buchner thus observed that a vigorous development of gas took place in the mixture. A further examination showed that the gas was carbon dioxide, and that alcohol was simultaneously produced in the juice. This was the basis of the extended researches which led to the discovery of the alcohol enzyme, which was successfully separated from the living cell (communicated first in 1897).

Buchner's process is as follows:—Fresh washed and strongly pressed yeast is ground with quartz and kieselguhr in a mortar. The cells are torn and broken open by the sharp sand, and the liquid absorbed by the kieselguhr. In a few moments the whole mass cakes together to form a dough. This is wrapped in a strong press cloth, and subjected to very high pressure in a hydraulic press, up to 90 kilogrammes per square centimetre. For every kilogramme of yeast about 500 c.c. of clear yellow or yellowish-brown juice is obtained. When the juice is mixed with a solution of saccharose, grape sugar or maltose, a strong frothing takes place within a few minutes, due to the development of carbon dioxide, and at the same time almost the same quantity of ethyl-alcohol is produced. By the addition of minute quantities of alkalies (potassium carbonate, disodium phosphate, etc.), the process of fermentation is quickened.

It can be shown that the fermentation is not caused by living cells remaining in the juice, for it is possible to add strong antiseptics like chloroform, thymol, or toluol,\* which would arrest every living function of the cells, or again the juice may be filtered free from germs through a porcelain filter, without destroying its activity. It might be supposed

\* On the other hand, mercuric chloride destroys the fermentative power of the juice.

that the fragments of protoplasm torn from the cells could be regarded as carriers of this power, and that the enzyme itself had not been separated. This cannot, however, be the case, for if the juice is treated with precipitants like alcohol-ether or acetone, the active substance is thrown down, and this, along with other precipitated substances, on drying, forms an amorphous and very stable powder which, on treatment with water, can once more be employed as an exciter of fermentation.

It has since been shown that high pressure is not essential. R. Albert has recently shown that by treatment of yeast with alcohol-ether, or, better still, with acetone in such a way that all the cells are destroyed, a very active powder can be prepared (*zymin*). The yeast is partially dried and soaked for a quarter of an hour in acetone (ten times its volume). It is then spread on filter paper to dry, washed with ether, and dried at 45° C. The preparation takes the form of a white powder. The powder, which consists of dead cells, that are still whole, produces almost immediate fermentation in a sugar solution. If it is washed with water, the water does not acquire any fermentative power. If, however, the cells are first disintegrated, it is possible by simple suction with a water pump to obtain a juice from which a precipitate is thrown down by means of ethyl alcohol, which can be dissolved in water and immediately produces a vigorous fermentation in a sugar solution. Whilst yeast that has been killed in the usual way does not retain any alcohol enzyme, it is possible by this method to fix the enzyme so that it remains intact in the dead cells.

These remarkable observations only permit of one explanation, viz., that living yeast cells are not essential for the production of fermentation, and that it is possible to separate an active enzyme from the disintegrated yeast cells which is soluble in water, and is much more resistant to antiseptics and other strong influences than the living cells, a property that it possesses in common with other enzymes. This substance, which is contained in pressed-yeast juice, Buchner named *zymase (alcoholase)*.

Buchner declares in very interesting fashion that this discovery proves that both Pasteur and Liebig were correct

in certain respects ; Pasteur in so far as zymase can only be produced by living cells, Liebig in so far as the fermentation is excited, not by living cells, but by a separate enzyme.

Thus, for the first time, we have a solid basis for a true theory of fermentation established by the study of that particular enzyme which brings about fermentation. A short *r  sum  * is given below of the properties of the enzyme, so far as they have been clearly defined in the short space of time succeeding its discovery. It must, however, be understood that very little is known concerning its chemical character.

Zymase cannot be regarded as consisting of living matter. It can be distinguished from invertase of yeast cells, which converts saccharose into fermentable sugar, by the fact that it does not diffuse through the cell wall.

If the juice is heated to 40°-50° C., a flocculent precipitate of albumen forms, and the clear liquid loses its fermentative power. Invertase has been identified in yeast-juice, and it must also contain an enzyme hydrolysing maltose and one hydrolysing glycogen, as it is capable of bringing about fermentation with these carbohydrates, neither of them being directly fermentable ; but, according to Hahn, it also contains a substance of importance, a proteolytic enzyme hydrolysing albumen. If a test tube containing fresh yeast-juice, and another containing juice that has stood for a week at room temperature in presence of toluol (to prevent the growth of micro-organisms), are placed in a water-bath at 40°-45° C., it will be found that in the former a strong coagulum separates out in a few minutes, whilst in the latter only a few flocculent particles are visible. The coagulable albumen, when kept for some time, disappears by a species of auto-digestion. Hahn has named the enzyme **yeast-endotryptase**. This enzyme reacts best in presence of acid, whilst the activity of zymase is improved by the addition of weak alkali. The presence of oxygen is advantageous to proteolysis. The enzyme can be isolated in a comparatively pure state, and is found in yeast cells. According to Hahn, it cannot be separated from quite normal cells, and such cells can only deal with those albuminoids which are forced through the cell walls.

Endotryptase has a powerful action on zymase, and even

when the juice is kept at a low temperature a marked loss in its fermentative power is observed in the course of a few days, owing to the influence of endotryptase. It is quite possible that it is this enzyme, more strongly developed, which attacks the enzyme of the yeast cells when they are exposed to unfavourable conditions. Buchner certainly believes that this accounts for the fact that yeast-juice prepared from one and the same species of yeast may contain very variable quantities of zymase. Zymase is extraordinarily sensitive both to variations in temperature and to the presence of strong alkalies. To protect the juice from the action of endotryptase large additions of cane sugar have been employed. Thus when mixed with 75 per cent. sugar solution the activity of the enzyme has been prolonged for several weeks.

As a result of a number of fermentations, Buchner notes that the fermentative power of 20 c.c. of yeast-juice with the addition of 8 grammes of sugar and 0.2 c.c. of toluol results in a yield of 1.87 grammes of carbon dioxide.

Compared with the fermentative power of fresh yeast, the action of the juice appears trifling. Thus, 1 grammie of good pressed yeast produces in an 8 per cent. cane-sugar solution 1.5 grammes of carbon dioxide in six hours at 30° C., whilst 20 c.c. of yeast-juice is produced from about 40 grammes of yeast, but it should not be forgotten that during the fermentation with living cells new zymase is constantly being produced, and that by no means all the existing zymase is extracted from the cells in the preparation of the juice.

To avoid the rapid decomposition of the juice, it may be dried in a vacuum at 25°-35° C. It forms a yellowish powder, which remains unchanged for a long time, and when dissolved in water displays an almost undiminished fermentative power.

In contrast to the action of weak alkalies, the addition of acid is prejudicial to the juice.

With regard to the best conditions of temperature, it has been shown that the highest fermentative activity is reached at 12°-14° C. The most favourable temperature for zymase undoubtedly lies higher, but it must be remembered that at the higher temperature endotryptase immediately comes into action and attacks zymase.

As to the concentration of the liquid, the largest amount of carbon dioxide is obtained by fermentation of liquids containing 30 to 40 per cent. of sugar, doubtless because in such concentrations the action of endotryptase is restricted. The fermentation lasts longer under these circumstances, and to secure a rapid fermentation, 10 to 15 per cent. of sugar should be employed, but under these conditions the action soon comes to an end.

Amongst the many attempts that have been made to isolate zymase from the juice, we may mention that by precipitation with alcohol-ether the whole of the zymase can be converted into a dry form without loss of activity. By the treatment of the dry substance with water and glycerine, the liquid, even when filtered, possesses the full fermentative power. This constitutes, therefore, a true solution of the active substance; a further treatment with alcohol-ether does not yield any increase of zymase in the precipitate.

The living yeast cells contain varying quantities of zymase. Thus the content of zymase often increases perceptibly in quiescent pressed yeast when kept at low temperatures. It is a remarkable fact that yeast cultivated in a strong sugar solution with inorganic salts exhibits a comparatively small amount of zymase at the moment of greatest fermentative activity with the greatest production of froth. If, however, the yeast is removed at this stage, washed, pressed, and stored for a few hours at a low temperature, it will be found that the zymase content has considerably increased. In the same way, yeast taken fresh from the brewery shows an increase of zymase in some cases after storing. In other cases no such increase is observed. These facts can be explained on the assumption that the endotryptase is influenced by the low temperature, even when the other conditions are favourable.

A short review of the chemical changes that take place during the fermentation of yeast-juice follows.

The first problem is to discover whether the phenomena caused by the addition of yeast-juice to sugar solutions are identical with those of alcoholic fermentation of sugar. The attempts to solve this have led to remarkable results. The chemical action of the enzymes already discussed—invertase,

maltase, lactase, diastase—consists in the hydrolysis of the polysaccharides into simpler compounds, the monosaccharides, an action which can also be produced by purely chemical treatment. Zymase is distinguished from these enzymes by bringing about the complete breaking down of the sugar molecule and the formation of new compounds, exactly like the alcohol enzyme of the living yeast cell. As is well known, this splits up sugar into almost equal parts of alcohol and carbon dioxide. This is also the case with zymase. A part of the sugar, however, is not converted into these products.

During the pressed-juice fermentation, glycerine is produced to the extent of from 3 to 8 per cent. of the fermented sugar; it is derived from the sugar. On the other hand, no succinic acid is produced. Acetic acid is formed in minute quantities, but somewhat more than in the fermentation with the living cell. This is probably due to the action of a special enzyme.

It is of great interest to know that lactic acid is often produced in the zymase fermentation, whereas in other cases the lactic acid originally present or that added to the liquor disappears. This observation suggests a possible solution of the way in which the sugar molecule is decomposed into alcohol and carbon dioxide. It is reasonable to suppose that lactic acid is an intermediate product, and that the zymase consists probably of two enzymes, one of which (zymase in the narrower sense) converts sugar into lactic acid; the other ("lactacidase") converts the lactic acid so formed into alcohol and carbon dioxide. The results could then be explained by supposing that an excess of one or other enzyme in the juice causes either the production or the decomposition of lactic acid.

It has been stated that part of the sugar is not decomposed into alcohol and carbon dioxide. This cannot be detected by the use of Fehling's solution, and it is certainly not present as reducing sugar. The experiments of Harden and Young have proved that a polysaccharide is formed by a synthesising enzyme present in the juice. By suitable hydrolysis this substance may be converted into reducing sugar.

The later work of Harden and Young has carried the investigation further. They found that by adding boiled

and, therefore, inactive yeast-juice to fresh juice, the activity of the latter is considerably increased. It follows that the juice must contain an enzyme which is stable at the boiling point, and another which cannot withstand this temperature, and that it is only in conjunction that they can exercise fermentative activity.

Buchner and Meisenheimer have obtained a pressed juice and also a stable preparation from yeasts fermenting lactose, which are capable of carrying on the fermentation.

It has not yet proved possible to isolate zymase, and nothing is yet known regarding its composition. Its properties may be summarised as follows:—It is soluble in water and dilute glycerine, and is not very sensitive to chemical reagents. In solution it is decomposed at 60° C. When yeast juice is kept at low temperatures (down to 0° C.), the zymase gradually disappears, whilst in a frozen condition it remains unaltered for some time. In a dry condition it may be stored for months with unaltered activity, and withstands a temperature of 110° C. It is precipitated along with albuminoids by treatment with alcohol, acetone, and ammonium sulphate. It can be dialysed with difficulty or not at all, and occurs in very variable quantity in the living cells according to their stage of development.

H. Fischer describes zymase as the *fermenting* enzyme to distinguish it from the other enzymes.

In addition to the enzymes already mentioned, the alcoholic, the hydrolytic (maltose-, cane sugar-, and glycogen-splitting), and the proteolytic enzymes, yeast-juice contains an oxidising, a reducing, a fat-splitting, a hydrogen peroxide-splitting, and a clotting enzyme.

The vitalistic view of alcoholic fermentation and of the other changes brought about by the yeast cell must be given up in the light of Buchner's and Fischer's discoveries, for it has been established that they can take place quite independently of the living cell. At the moment this has no direct or important bearing upon the fermentation industry. The particular action of selected species of yeast on the individual substrata, wort, must, etc., is undoubtedly a product of the action of the complicated forces in the cell, of which only a

small number are known. To secure the results required in practice, it is still necessary, therefore, to make use of living growths.

### The Enzymes of Yeast.

In addition to the general sketch included in the foregoing chapter on the enzymes occurring in fungi, a few particulars must be given regarding their special relations to yeast.

**Invertase** is commonly found in all species. It is prepared by treatment of the yeast with alcohol or ether, or by drying and heating to 100° C. The enzyme is then extracted with water or glycerine, and precipitated with alcohol; the precipitate is afterwards dried. It hydrolyses cane sugar, which is split into one molecule of glucose and one of levulose, and it is only after this decomposition that yeast can ferment sugar. The optimum temperature of invertase is about 55° C., and it is destroyed at 75° C., but in a dry state it can stand much higher temperatures. It is resistant to small doses of antiseptics, and to vigorous action of the proteolytic enzyme of yeast. Its action is increased by very dilute acids, but considerably diminished by treatment with alkali.

**Maltase** also occurs in many yeast species. It decomposes maltose into two molecules of glucose. It has its optimum at 40° C., and is destroyed at 55° C. According to Bokorny and others, it is much more sensitive to chemical reagents than invertase.

**Melibiase**.—By careful treatment of raffinose with dilute acid, it is hydrolysed into levulose and melibiose. The latter is decomposed by melibiase, which, according to Bau, is usually present in bottom-fermentation yeast, but is often absent in top-fermentation yeast.

**Lactase** decomposes lactose into one molecule of glucose and one of galactose. It is only after this enzyme has reacted that lactose can be fermented. It has been found in a number of yeast species.

According to van Laer, P. Lindner and others, several yeast species are capable of fermenting dextrin. It is assumed that the fermentation is preceded by a similar hydrolysis to

those previously mentioned. Such an enzyme (**amylase**) has been detected in a few moulds.

Amongst the enzymes found by Buchner and Hahn in yeast-juice, the proteolytic enzyme or **endotryptase** plays an important part in the life of the cells, especially during the so-called auto-fermentation. Its presence was mentioned in the early literature of the subject. Its optimum is at 40°-45° C., and it is completely destroyed by one hour's heating at 60° C. In a dry condition it is more resistant. It can withstand the action of weak antiseptics, and weak acids. Neutral salts react favourably. Saccharose, even in a 5 per cent. concentration, restricts its activity, and in a 35 per cent. concentration it is completely arrested. Will examined a series of yeasts, and proved that the rate at which they liquefy gelatine is variable, and that those that act rapidly are also those that require a free supply of oxygen.

Reducing enzymes are also found in yeast cells, and amongst these must be classed the enzyme which converts sulphur into sulphuretted hydrogen.\* According to Nastukoff, Osterwalder, Schander, Will, and others, it occurs in very different degrees of activity in the various yeast species.

A clotting enzyme in yeast has been detected by Rapp.

#### The Action of the *Saccharomycetes* and similar Fungi on Carbohydrates and other Constituents of Nutritive Liquids.

##### Diseases in Beer.

The first decisive proof that species of *Saccharomyces* may produce very different reactions on the nutritive liquid was given by means of pure cultures of yeasts prepared by E. C. Hansen in 1883, and afterwards by the author.

\* Both in beer and wine a formation of sulphuretted hydrogen occurs (in the latter case called "Böckser"), more particularly at the end of the fermentation. As both the grapes and the wine and beer casks are treated with sulphur, it can find its way into the liquid, but even in the absence of free sulphur or sulphur compounds, the albuminoids of the nutritive liquid and the contents of the cell protoplasm may provide material for the formation of sulphuretted hydrogen. Certain experiments appear to indicate that a diseased condition of culture yeasts may be connected with this fact.

Hansen's epoch-making researches on disease yeasts proved that amongst the wild yeasts there are groups which bring about detrimental changes in beer, whilst others proved to be harmless. Amongst the former there are some which impart a bitter taste and disagreeable odour to beer (*Sacch. Pastorianus I.*), usually without producing turbidity, whilst others (*Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*) only fully develop their activity at a late stage of the secondary fermentation, and then make the beer turbid. This effect is due to the abundant yeast deposit formed a comparatively short time after the finished beer has been drawn off, which rises at the slightest movement of the liquid. These disease yeasts cannot produce turbidity if they only come in contact with beer at the end of the principal fermentation. It is possible, however, if the beer comes in contact with the two species after storing, that an infection with young cells of *S. ellips. II.* may produce turbidity. The disease yeasts which influence the odour and flavour of beer are only of importance when they occur at the beginning of the principal fermentation. The chief danger lies in the pitching yeast. Weakly fermented beer is much more liable to attack than other beer. Hansen's observations on the disease yeasts have been confirmed and extended by Grönlund, Will, and others. Becker made the interesting observation that certain wild yeasts, which impart a bitter taste to beer, are capable of influencing the attenuation when mixed with culture yeasts. The fermentation is increased and in certain cases extends over a longer period than with the pure culture yeast. Wild yeasts can also bring about disturbing effects in top-fermentation breweries. For instance, according to de Bavay, the "summer-cloud" of Australian beer is caused by a *Saccharomyces*, which causes turbidity, and imparts a bitter and slightly acid taste. In English high-fermentation beers the author found yeasts of the *Saccharomyces anomalus* type which produced turbidity; in weakly-fermented, Danish high-fermentation beers, *Torula* species, having similar properties occur. Similarly van Hest found species of *Torula* producing turbidity in top-fermentation Dutch beers. Chapman found that *Sacch. Past. I.* occurs in English beer, and gives the well-known bitter taste known as

“yeast bite.” Frew observed that the “stench” in English beers, which have undergone secondary fermentation, due to sulphuretted hydrogen or a similar substance, is derived from a special wild yeast, *Sacch. foetidus* I. It is well known in practice that *S. ellips.* II. and other species can produce diseases even when the beer is first infected in the storage casks, transport casks, or bottles. Reference must be made to the fact that mixtures of culture yeasts, each capable of producing a good product, may, according to Hansen, produce diseases in beer. By the use of mixtures for the pitching yeast it was found that the species present in smaller quantity rendered the beer more liable to turbidity, in comparison with fermentation carried on with the leading species alone. Even when the two species were separately applied, and the beers mixed for the first time in the storage casks, similar phenomena were observed.

Pichi has found species producing disease in wine.

In the storage casks of lager beer, Lafar found a budding fungus of the *Mycoderma* type, which produced acetic acid.

Just as the moulds react differently upon various carbohydrates, so it has been shown by the exact researches of Hansen and others that the yeasts exhibit pronounced characteristics. In addition to the true *Saccharomycetes*, *Mycoderma cerevisiae*, *Sacch. apiculatus*, the *Torulas*, and *Monilia*, are reviewed in the following paragraphs:—

Hansen’s six *Saccharomycetes* (*Sacch. cerevisiae* I., *Sacch. Pastorianus* I., II., and III., *Sacch. ellipsoideus* I. and II.) behave as follows:—They all develop invertase; they convert saccharose into invert sugar and ferment the latter; they ferment maltose and dextrose, but not lactose. All the bottom yeasts used in practice react similarly with these four sugars.

*Sacch. Marxianus*, *Sacch. Ludwigi*, and *Sacch. exiguum* do not ferment maltose and lactose; they invert saccharose and ferment nutritive solutions of invert sugar and dextrose.

*Sacch. membranæfaciens* and *Mycoderma cerevisiae* possess no inverting enzyme, and do not ferment any of the four sugars.

*Sacch. apiculatus* does not invert saccharose, and of the

four sugars it only ferments dextrose. It only induces, therefore, a feeble alcoholic fermentation in beer-wort.

Amongst the *Torulas* there are many which do not secrete invertase, are incapable of fermenting maltose, and only yield about 1 per cent. by volume of alcohol in beer-wort. Other species invert saccharose. In nutritive dextrose solutions the different species induce a more or less vigorous fermentation.

*Monilia candida*, although possessing no inverting enzyme soluble in water, ferments saccharose, maltose, and dextrose. It ferments beer-wort, but at ordinary room temperature it only yields the higher percentages of alcohol at a much slower rate than the *Saccharomycetes*.

In milk, various budding fungi have been found. Of these, Grotenholt and the author have described certain *Saccharomyces*; Duclaux, Adametz, Kayser, and Beijerinck several non-*Saccharomyces*. They all decompose lactose. Fermi found that certain red and white yeasts exercise a diastatic action. Morris arrived at similar results in experiments with pressed yeast.

If we now review all these different properties of the *Saccharomyces*, we shall see that they fall into two groups:—

I. Those which possess an inverting enzyme and induce alcoholic fermentation. This group is further subdivided into:—

(a) Those which not only ferment saccharose and dextrose, but also vigorously ferment maltose (the six species first described by Hansen, and the yeasts employed in the brewing industry);

(b) Those which ferment saccharose and dextrose, but not maltose (*Sacch. Marxianus*, *Ludwigii*, and *exiguus*).

II. Those which do not possess any inverting enzyme, and do not induce alcoholic fermentation (*Sacch. membranæfaciens*).

The budding fungi which do not form endospores (non-*Saccharomyces*) show the most varied characters with reference to the properties of inversion and fermentation.

I. The great majority do not ferment maltose. Many of these induce a more or less vigorous fermentation in solutions of dextrose and invert sugar. Some *Torulas* invert saccharose.

and many possess no inverting ferment (*Mycoderma cerevisiae*, *Torulas*, and *Sacch. apiculatus*).

II. The *Torula novae carlsbergiae*, and a few of the species isolated by Will, ferment maltose. One species (*Monilia candida*) resembling *Torula* ferments maltose, as well as both saccharose and dextrose. It contains no inverting enzyme soluble in water.

The lactose-fermenting *Saccharomyces* and *Torulas* demand a special classification.

When we consider the behaviour of these fungi in the fermentation industries, it is at once seen that it is only in the genus *Saccharomyces* that species occur which rapidly and vigorously ferment maltose. The yeasts for breweries and distilleries must, therefore, be selected from the true Saccharomycetes. The non-Saccharomycetes, the great majority of which cannot ferment maltose, are scarcely capable of playing any important part in these industries; on the other hand, they may be employed in the manufacture of wines from grapes, currants, and other fruit, since several are able to induce just as vigorous a fermentation in solutions of dextrose and invert sugar as the Saccharomycetes.

It is, therefore, of the utmost importance that a suitable species should be selected.

Amongst the carbohydrates synthetically prepared by E. Fischer, isomaltose may be mentioned. For some time it has played a great part in the literature of the subject. As is well known, he discovered this sugar in the products of the reaction at a low temperature of hydrochloric acid on grape sugar, and it received the name of isomaltose because it appeared to have a constitution similar to that of maltose. The sugar is known only in the form of an osazone. Even the existence of Fischer's isomaltose has been questioned, because it was regarded as impure maltose. By a fresh investigation, however, Fischer succeeded in proving biologically that this sugar is sharply distinguished from maltose by the fact that isomaltose is neither fermented by fresh yeast nor split up by the enzymes of yeast, and he asserts that it is only possible to differentiate with certainty between the two sugar species in this way.

The different action of the *Saccharomycetes* on the same nutritive liquid (wort or must) under identical conditions, has been further studied by Borgmann, Amthor, and Marx.

According to Borgmann, the chemical reactions brought about in wort by the two Carlsberg bottom yeasts, No. 1 and No. 2, show a striking difference. These two species—which had been in use for some time in the fermenting room, and were still practically pure—were employed for pitching two fermenting vessels containing wort from the same brew; the fermentation took place under conditions which enabled a true comparison to be made, and the resulting beer was stored as usual. The differences in the chemical reaction were especially noticeable in the proportion of free acid. Thus:—

	No. 1.	No. 2.
Free acid (calculated as lactic acid),	0.086	0.144 per 100 c.c.
Glycerine,	0.109	0.137 "

As a result of these experiments, Borgmann pointed out that the ratio between the alcohol and glycerine in these two beers differs from that previously found in beer, the ratio obtained from previous analyses being:—

	Alcohol.	Glycerine.
Maximum,	100	5.497
Minimum,	100	4.140

Whilst the Carlsberg beers gave the following ratios:—

	Alcohol.	Glycerine.
No. 1, .	100	2.63
No. 2, .	100	3.24

It must be admitted, as Borgmann observed, that good beer may be produced by a method not open to criticism, in which the ratio of alcohol to glycerine may sink below the previously-admitted minimum.

A series of the eight different species of *Saccharomyces*, and amongst them six culture yeasts, all in absolutely pure cultures, were examined by Amthor with reference to their chemical action on beer-wort. The fermentations were conducted in Pasteur flasks of one litre capacity under identical conditions, and formed two series, one corresponding to the primary fermentation in the brewery, and the other to the secondary

fermentation. The amount of alcohol, extract, the specific gravity, attenuation, glycerine, nitrogen, reducing substance, and degree of colour, were determined in the fermented worts. The tables show palpable differences in the chemical reactions brought about by the different species. The percentage of alcohol varied within the limits of 4.34 and 6.02 by volume (3.55 to 5.94 at the end of the primary fermentation), the extract from 8.27 to 11.23 (8.49 to 11.61 at the end of the primary fermentation), the attenuation from 36.7 to 53.3 (28.8 to 52.1 at the end of the primary fermentation); the percentage of glycerine showed very striking differences, and fluctuated between 0.08 and 0.15; likewise the amounts of nitrogen, of reducing substance, and to some extent even the colour intensity, showed considerable variations.

Hiepe drew some interesting parallels between the behaviour of a number of culture yeasts and wild yeasts with regard to the sugars. For this purpose he instituted fermentations in sugar solutions containing yeast decoction. He took out the first sample five minutes after the fermentation has been induced, and then fresh samples every day, till the fermentation had subsided. In each sample the amount of (1) inverted sugar, (2) fermented extract, (3) fermented dextrose, and (4) fermented levulose was determined. In these four respects well-marked, specific differences developed in the course of a day. Thus, in five minutes an English high-fermentation yeast had inverted 1.95 per cent. sugar, whilst a low-fermentation yeast from the author's collection had inverted 58.85 per cent. A complete inversion of the sugar with two low-fermentation brewery yeasts took place in the course of about twenty-four hours, whilst in the case of *Sacch. exiguis* this reaction required eleven days; for the other species the time required lay between these two limits. The detailed tables, given by Hiepe, show that the successive fermentation of the total quantity of extract, as well as that of the two sugars, takes place according to a scale peculiar to each individual species. A glance at the numerous details of the experiments further shows that the fermentation of dextrose, as a rule, begins much more vigorously than that of levulose; but whilst the fermentation of the former reaches its maximum

on the second day, the fermentation of laevulose does not reach its highest activity until later, in some species even as late as the fifth day; by slow degrees the proportionate amounts of sugar fermented approach each other, and finally both sugars disappear simultaneously.

The yeast species also behave differently with regard to the amount of acid produced in the nutrient liquid. From this point of view Prior examined the fermentation products of a number of brewery yeasts and wild yeasts in hopped wort, and found that the amounts of acid formed varied from 4.7 to 10 c.c. of decinormal caustic soda solution per 100 c.c. of fermented wort; the fixed organic acids varied from 2.1 to 5.4 c.c., the volatile organic acids from 2.1 to 5.8 c.c. The evidence shows that, in culture yeasts, the amounts of fixed organic acids usually exceed those of volatile acids, whereas in Hansen's wild yeast species (*Sacch. Pastorianus I., II., and III.*, and *S. ellipsoideus I. and II.*) the reverse is the case, the volatile acids exceeding the amount of the fixed acids; this is specially the case with *Sacch. Pastorianus I.*

A large number of Saccharomycetes occurring in must—absolutely pure cultures of which were prepared by Hansen's method—were examined by Marx in 1888 both botanically and with reference to their chemical action on the nutritive liquid. They showed distinct differences in fermentative power, and in their capacity for producing volatile substances which impart a special bouquet to wine, and finally in their power of resistance to different acids and to high temperatures.

Amthor subsequently investigated a number of absolutely pure cultures of wine yeasts, and detected typical differences with regard to the time taken by the fermentation, as well as in the chemical composition of the wines. Similar results have also been obtained by Jacquemin, Rommier, Martinand, and Rietsch in France; Müller-Thurgau in Switzerland; Wortmann and Nathan in Germany; Mach and Portele in Austria; Forti and Pichi in Italy; some of the comparative experiments conducted by these authors having been carried out on a large scale.

The most thorough and extensive investigations into the different behaviour of wine yeasts with regard to must are due

to J. Wortmann. He states, as the general upshot of his investigations, that the differences in the divers types of genuine wine yeast are sometimes so great that they can be detected merely through the chemical analysis of the products of fermentation or metabolism ; in other cases, however, they are of such a kind that we can only convince ourselves directly of their presence by their odour and flavour. Every type of yeast shows some individual peculiarity more or less characteristic in its action on any must, regardless of its nature or origin.

The number of yeast cells formed in a given must, apart from the nutritive contents of the must, depends on the specific power of propagation of the chosen type ; on the other hand, it is in itself independent of the origin of the particular must. In any given must, whether it be an excellent or an indifferent nutrient medium for the wine yeast, one yeast type will multiply more freely than another.

An extensive comparison of the amount of extract contained in a number of wines fermented with three different yeast species showed that in the same must the "Würzburger" yeast consumed the smallest quantity of extract ; next came the "Johannisberger," whilst the "Ahrweiler" yeast used up the largest amount of extract, and, accordingly, left the smallest residue in the wine.

The specific activity of wine yeasts is clearly brought out in the formation of glycerine, which has a predominant influence in determining the flavour of wine. The three species mentioned above were compared in a large number of musts of different origin, and, on the average, the Würzburger yeast formed more glycerine than the other two ; of these, the Johannisberger yeast was superior to the Ahrweiler, which, as already stated, ferments the extract most vigorously.

The difference observed between the chemical activities of these species was emphasised by the fact that the Würzburger yeast had multiplied most feebly. This example, amongst others, shows that the alcoholic fermentation of must is independent of the formation of glycerine. It is, therefore, impossible to establish a definite relationship between the contents of glycerine and alcohol in wine.

Both the percentage of nitrogen and of ash proved to be different in wines fermented with the three kinds of yeast.

The acid content was highest in the wines fermented with Würzburger yeast, and practically equal for the other two kinds.

In accurate comparative experiments, a large number of species differed widely with respect to the amount of alcohol produced in the liquid; those yeasts having the shortest fermentation period yielded the smallest percentage of alcohol, and conversely.

With regard to the bouquet in wine, Wortmann and Müller-Thurgau distinguished between that which originates in the grape—"grape bouquet"—and that which is produced as a result of the activity of yeast—"fermentation bouquet." In some wines the grape bouquet is so strongly developed that the fresh bouquet formed by the action of yeast on some of the substances present in the grape plays only a subordinate part in determining the character of the wine. In other wines where the grape bouquet is not so strongly developed the fermentation bouquet may have a great influence on its character. The result of applying a pure culture of a wine yeast will, therefore, differ with different wines. In the first group of wines the influence must be regarded as an indirect one in that the pure culture suppresses foreign organisms, which might mask the true grape bouquet, whereas the fermentation bouquet will have but little influence on the wine. In the fermentation of such wines the best results will be obtained by using yeasts from the locality. It is quite otherwise with the second group of wines. With such musts possessing no outstanding characteristics, the application of specially selected yeasts will exercise a directly favourable influence on the flavour and on the whole character of the wine. The long experience of the author has fully established the fact that both the quantity and the variety of the fermentation bouquet may be increased by such means.

The fermentation bouquet differs with each yeast species, but neither its quality nor quantity stands in direct relation-

ship to its fermentative power, but a certain uniformity appears to exist among the yeast species in their action on any given wine.

A complete alteration of the fundamental character of a wine by the use of pure cultures of wine yeasts is impossible, but by a suitable selection of species and the right method of application important advantages can be secured in every case. The same applies to the different kinds of fruit wines, which may be fermented both with grape wine yeasts and with pure cultures of the species growing on the respective fruits.

Kayser compared the chemical properties of several types of wine yeast, and found that the formation of volatile acids at higher temperatures differed for each species. Thus, with a rise in temperature, the quantity of these acids increased in one species and decreased in another.

Forti, basing his conclusions on comparative experiments with wine yeast, has drawn attention to the existence of typical differences in the fermentative power of the species, power of resistance to high temperatures, and both quantity and quality of the nitrogenous constituents required in the nutritive liquid. According to his view, there is a well-marked distinction in the character of the fermentation produced by yeasts in the primary or vigorous fermentation, on the one hand, and those of the secondary or quiet fermentation on the other.

The numerous investigations carried out continuously since 1884 in the author's laboratory with pure cultures of yeasts, as applied in the various branches of the fermentation industry, have furnished ample opportunity for collating experience relating to the chemical activity of species, and to their respective powers of retaining their peculiarities intact during storage, a matter of importance to every branch of the industry. Numerous instances have been met with in which even feebly-pronounced characters, manifested through taste or smell, remain inherent after several years' preservation of the growth; they may be restored by suitable development of the culture under favourable circumstances.

**The Products of Alcoholic Fermentation.**

It has already been stated that saccharose can only be fermented after the intervention of invertase has caused absorption of water and decomposition into glucose and lactose. The same holds good with regard to maltose, which is split up into two molecules of glucose. In a similar way lactose is split up by certain species of yeast before alcoholic fermentation takes place. Other sugars (the hexoses) are directly fermentable. Of these, the commonest is glucose or dextrose (grape sugar), which is fermented by every known species of alcoholic yeast. This also applies to lactose or fructose, which is so widely distributed in the vegetable kingdom, and usually occurs in conjunction with dextrose. Invert sugar is a mixture of the two.

The principal product of fermentation is alcohol, more particularly ethyl alcohol. In 1815 Gay-Lussac first established the true character of the reaction when he showed that cane sugar (more correctly grape sugar) gave 51.11 per cent. of alcohol and 48.89 per cent. of carbon dioxide on fermentation. Pasteur showed that by-products always occur, and that part of the sugar is utilised for the nutrition of the yeast, so that it is never possible to convert the whole amount of sugar into alcohol and carbon dioxide. Pasteur's results were 48.3 per cent. of alcohol and 46.4 per cent. of carbon dioxide, which agrees well with recent determinations, showing that practically equal quantities of alcohol and carbon dioxide are formed. It has already been stated that in all probability lactic acid is formed as an intermediate product in the fermentation, as shown by Buchner and Meisenheimer's work on the action of yeast juice.

Rayman and Krčíš proved that beer which had been subjected to fermentation with absolutely pure cultures, and kept for some years at the usual temperature, contained only ethyl alcohol, but when air was introduced and the yeast formed a film, the alcohol was decomposed into carbon dioxide and water.

Glycerine occurs in varying quantities, and, according to Wortmann and Laborde, this does not depend entirely on the

decomposition of the nutritive liquid, but more particularly upon the yeast species. Its production is favoured by a high temperature of fermentation, and by a greater sugar concentration, or in general by the use of a rich nutritive fluid. It is almost impossible to give limits for the proportion of alcohol and glycerine. In wine fermentations the formation of glycerine varies usually from 2.5 to 14 per cent. of the amount of alcohol produced, whereas in beer it represents only 1.65 to 4.3 per cent. of the alcohol. By fermenting saccharose with zymase Buchner and Rapp obtained even smaller quantities of glycerine.

Succinic acid is another by-product which varies in quantity. According to Rau, the quantity increases with increasing temperature, and apparently the composition of the nutritive fluid has no influence on the result.

Lactic acid is always found as a by-product in fermentations carried on in the absence of living cells.

Prior's detailed researches prove that the different races of yeasts produce very varying quantities of volatile and non-volatile acids. He found that acetic acid was a constant product of fermentation.

Formic acid is produced, according to Rayman and Kruis, by the oxidising action of yeast on the albuminoids of the nutritive liquid.

Aldehyde (acetaldehyde) also occurs regularly and must be regarded as an intermediate product between the fatty acids and the alcohols. Rayman and Kruis proved that, especially in the case of distillery yeasts, considerable quantities are formed when free access of air is permitted, and the surface of the fermented liquid is then covered with a film of yeast. They assume that acetaldehyde is produced by oxidation of ethyl alcohol.

Methyl alcohol, often found in bacterial fermentations, may also be developed during a yeast fermentation, especially by the fermentation of glucosides present in fruit juices. It also appears possible that propyl and butyl alcohol may be produced in a normal alcoholic fermentation, the former from lactic acid. Especial interest is attached to the presence of amyl alcohol (isoamyl alcohol), which forms the main constituent

of fusel oil; according to Rayman and Kruis, it is produced in larger quantities at high temperatures and in the absence of air. Amyl alcohol is freely produced in liquids containing grains which have been treated with sulphuric acid. According to Ehrlich, fusel oil is formed in the ordinary growth of yeast from leucin and isoleucin, two cleavage products of albumin. It appears to be produced also in the auto-digestion of yeasts.

We must also record the production of acetic ether and other volatile and non-volatile ethers which help to impart the particular character to the fermented liquids.

#### Auto-fermentation.

Pasteur's researches indicated that yeast is capable of forming alcohol and carbon dioxide under certain conditions, even in the absence of sugar from the surrounding liquid. By boiling yeast with dilute sulphuric acid, he prepared a fermentable sugar which he believed to be derived from the cell-wall.

Salkowski has proved that in reality glycogen plays a part in auto-fermentation. As glycogen can be fermented by Buchner's yeast-juice, it is concluded that the yeast cells contain an enzyme which can hydrolyse glycogen before it is fermented. Salkowski states that by treatment with chloroform-water glycogen is split up, but auto-fermentation does not take place. C. J. Lintner found that sodium chloride has a similar action, and in the presence of chlorides of sodium, calcium, magnesium, or ammonium no such fermentation takes place. On the other hand, sodium sulphate and magnesium sulphate react favourably. It is necessary, therefore, in fermentation experiments to include in the calculation the amount of alcohol and carbon dioxide produced from the yeast cells.

It is not only the carbohydrates, but also the nitrogen compounds that are gradually resolved in the yeast cell. Hahn proved the presence of a proteolytic enzyme in Buchner's yeast-juice, and it is known that yeast has the power of liquefying gelatine. In auto-digestion Kutscher and Lohmann detected a number of cleavage products of the proteins, more especially guanin and adenin, and also leucin,

ammonia, etc. Their experiments were carried out in presence of toluol to prevent an infection with bacteria. The process of auto-digestion begins if yeast is kept for a long time at a high temperature without nitrogenous food.

In practice, auto-digestion may take place in the manufacture of pressed yeast where decomposition of the yeast is frequently encountered without the occurrence of any bacterial infection. In this case, owing to lack of nourishment, the cells gradually resolve their albuminoids, and doubtless a proteolytic enzyme is simultaneously secreted. The yeast mass is then more readily exposed to infection by bacteria. Cells rich in glycogen appear to be less liable to such a decomposition.

#### **Fermenting Power ; Fermentative Energy ; Raising Power.**

The work carried out by yeast can be distinguished under three heads :—The activity of the enzymes ; metabolism ; synthesis of material.

The activity of the enzymes is of a sugar splitting, hydrolysing, and proteolytic character. As proposed by Neumann Wender, the fermenting power of yeast may be expressed in terms of the quantity of sugar which is split up at a given temperature in unit time by unit quantity of yeast. The fermentative energy may be defined by determining the time required within which a given quantity of sugar is decomposed by unit mass of yeast under special conditions. The " raising power " is a function of the carbon dioxide formed by pressed yeast, whereby the dough is raised. Carbon dioxide is developed during both true and auto-fermentation. A true and practicable determination of the " raising power " of baker's yeast can only be carried out in so far as it is possible to prepare a kind of normal dough.

#### **The Biological Relationships of Yeast.**

The problem of the occurrence of yeast in nature was raised as soon as its vegetable character had been established. The first researches on this question were undertaken by Brefeld in 1875, who arrived at the result that the yeasts are very

widely distributed in nature, and that their germs are present in atmospheric air, in dust, and in vegetable matter, and that their breeding places are specially to be sought in the excrement of herbivorous animals. Here they can exercise their fermentative power. It will be seen from what follows that this view can no longer be accepted. It is true, as the author has proved by his own investigations, that the excrement of herbivorous birds contains numerous budding fungi, and amongst them *Saccharomyces*, but their breeding places must be sought in quite a different direction.

In 1876 and 1879 Pasteur published complete memoirs regarding the occurrence of yeasts on grapes, and stated that they were to be found only on ripe grapes. At the same time he did not succeed in answering the important question as to where the yeast fungi found a habitat during the remaining part of the year. He expressed the view that *Dematium pullulans*, which is found everywhere on grapes, lives through the winter in the form of thick-walled and coloured resting-cells, and produces new yeast cells in the following summer, but it is now recognised that these budding cells are not wine-yeast cells. On the other hand, it was shown by the author in 1895 that other mould forms occurring on grapes, which resemble *Dematium*, but do not possess thick-walled resting-cells (Fig. 45), produce internal spores which develop budding *Saccharomyces* cells. What part these moulds play in the preservation of the yeast vegetation has not yet been determined.

Great uncertainty still existed regarding the most important question as to where the yeast remained during the different seasons of the year. It was established for a single species by E. C. Hansen in 1880-81, and his further researches, of a very detailed and fundamental character, have cleared up the question for so many other species that this important phase of the biology of yeasts is now fully understood. The researches of Hansen were first carried out on the small lemon-shaped yeast-fungus *S. apiculatus*, which always appears in the earliest stage of wine fermentation. By a microscopical examination and culture experiments it was shown that during the summer months the organism appeared in vast quantities with the

ripening of the sweet juicy fruits (cherries, gooseberries, strawberries, grapes, plums, etc.). On the other hand, it was only quite exceptionally that they were found on the unripe fruit. As the organisms were found vigorously budding on the ripe fruit, but never, or only very rarely on other fruit, and on the leaves, branches, etc., the fact may be accepted that these ripe fruits act as a true host to *S. apiculatus*. This was further established by the observation that they are to be found without exception in the soil under cherry and plum trees, vines, and other fruit-bearing trees upon which the organism grows, but that they are extremely seldom found in samples of soil taken in other localities of a most varied character. The fruit falls to the ground, and the rain carries the fungus into the soil; the problem, then, is whether it is able to winter there. The answer was obtained in two ways. First, numerous samples of soil were taken during the course of the winter and spring at these places, and in the vast majority of cases these gave a vigorous growth of the organism in wort. Secondly, cultures of *S. apiculatus* were placed with every precaution in the earth, and allowed to remain throughout the winter. They were removed in the spring and early summer, and culture experiments proved that the organism was alive in every sample. In this way it was established that the organism is able to winter in the earth, just as it had been previously shown that it only occurred in the soil at these particular localities. In later experiments of Hansen's, vigorous growths of the organism were placed on the surface of the soil in well-sealed Chamberland filter tubes. Three years later the contents of these tubes were introduced into sterilised wort, and a vigorous growth of the organism developed. The cycle of operations may, therefore, be spread over more than one year.

It still remained to be proved whether the earth is the true habitat in winter time. This was carried out as follows:— Hansen examined dust in a great variety of places from January to June, and also the dried fallen fruit of many trees, and lastly, many kinds of excrement. These analyses gave a negative result, and thus furnished the desired proof. The soil under the particular fruit trees must, therefore, be regarded

as the true winter habitat of the fungus. It preserves its usual appearance throughout the long winter time, and is then carried up into the air by the combined agency of insects and of wind, and by these means of transport it is distributed from fruit to fruit.

It is obvious that during the period when a large number occur on ripe fruit the currents of air may carry the fungus to other places, and also on to unripe fruit. Hansen stated in his first memoir that the rare occurrence on unripe fruit must be due to the fact that the organism quickly dies off, partly through want of nourishment, and partly through the drying up of the cells. He subsequently proved by experiment the correctness of this view. He distributed both old and young cells in water, and placed them either in a thin layer on an object glass or on a tuft of thinly spread cotton-wool; thus allowing evaporation to go on while the cells were protected from the sun. In less than twenty-four hours the whole of the cells were killed. It is quite obvious that the individual cells spread over the surface of unripe fruit are exposed to more unfavourable conditions than in his experiments. If, however, thicker layers of the cells are covered by cotton-wool or filter paper, they remain living just as they do in the soil for a long time. Thus they live for more than eight months in filter paper.

It was then possible for Hansen to demonstrate that the greater number of yeast species must pass through a similar cycle in nature. Their most important breeding places are the sweet juicy fruits. Their winter habitat is the soil, and they are carried by wind, rain, insects, and other creatures on to the fruit. They then multiply once more on sweet fruit, and obviously more particularly where the juice oozes out from the fruit. Hansen further found that these yeast species often occur in the ground at places far removed from orchards, where *S. apiculatus* can no longer be found.

Müller-Thurgau arrived at the same results as Hansen with regard to *S. apiculatus* during an examination of the wine species. He found that grapes are their chief breeding places, and that their presence may be distinguished in the soil throughout the year. On the other hand, they seldom occur

in the air. He further proved that the wine-yeast cells may occur in soil at a depth of from 20 to 30 cm.

In 1897, Wortmann's researches, recorded in his work on the preparation of wine, were directed to determining the behaviour of wine yeasts in soil at different seasons of the year. The experiments were continued for two years, and consisted in taking samples of soil every fourteen days from one and the same part of a vineyard. By sowing the soil in sterile must, he obtained an idea of the vegetation. His main observation was that directly after the vintage (in November and also in December) the samples of soil developed a growth of yeast in must so rapidly that no other fungi were able to develop. In January, February, and March also a development of yeast was always obtained from the samples, but it occurred more slowly. In the spring and summer the conditions were always less favourable, and a longer period elapsed before fermentation began. Some samples, indeed, gave no yeast development, but only other organisms. The least favourable conditions were observed in the late summer (August and September), but from the time the grapes began to ripen, a vigorous growth was again observed in the flask. Wortmann concluded that while the wine yeast remains in the soil its nutritive state is of the greatest importance. The vegetation is most vigorous during the early stages, when it has been enriched with cells fresh from the grapes—*i.e.*, in the autumn, winter, and the beginning of spring—whereas during the summer, the most favourable period for vegetation generally, its power is constantly diminishing, the cells having drawn upon their reserve material. According to this view, the yeast is dependent upon its own body-material during its habitat in the soil. The lower temperature ensuing after the vintage allows metabolism to go on so slowly that it enables the cells to maintain life throughout the winter and spring.

At the beginning of summer, with increasing temperature, the cells rapidly assimilate the remainder of the reserve substance, and consequently die off slowly. The cells that are still alive are weakened, and the samples of soil, therefore, give a very feeble growth in the flasks. The cells are continuously carried by insects and other means from the soil to

the vegetation, and those which light upon the grapes when they are ripe find full nourishment, and produce a new vigorous growth. Wortmann was able to confirm Müller-Thurgau's observation that no wine yeasts are to be found in a vineyard which has not been worked for a long time; they are gradually killed out by exhaustion. In those wine districts where the culture of grapes has been continued for centuries, the yeast cells which are brought from the soil when the grapes are ripe adapt themselves more and more to the excellent nutrient material, and in this way specially good races of wine yeasts are developed.

In 1903 and 1905 Hansen obtained results which differed from those of Wortmann in one important point relating to the condition of yeast cells during their abode in the soil which the latter regarded at a state of starvation. This new and very detailed research led to the result that elliptical and *Pastorianus* forms of *Saccharomyces* (but not *S. apiculatus*) are to be found throughout the year in all kinds of soil in the neighbourhood of Copenhagen. Their number diminishes, however, at a distance from the orchards. A similar condition of things was found by examining soil in the Harz Mountains and in the Alps. The soil in vineyards is specially rich in yeast species, and the greater the elevation the smaller is the number of organisms found. Above a certain height no organisms are found.

The reason for this wide distribution lies, as Hansen showed, in the fact that, in addition to the normal breeding places for yeast, there are others which he called secondary breeding places—e.g., aqueous extracts from fruit and other vegetable matter and from excrement. In the former, the cells multiply very rapidly, in the latter, feebly or not at all. If yeast cells from sweet juicy fruit and from the upper layers of soil, where they form spores, are carried by insects or by wind to distant places, they may, unlike *S. apiculatus*, maintain life even when dried, on account of their greater power of resistance. In the same way they can multiply more readily in soil in the aqueous extracts already referred to, and may even preserve life for a longer period in presence of nothing but moisture. Thus the fact is fully explained that the larger species occur much more widely distributed throughout the soil than the

small lemon-shaped wine yeasts. *S. anomalus* and *S. membranæfaciens* are especially resistant to the effect of drying. They are, therefore, found at great distances from the primary habitats. In this way the fact may also be explained that fewer yeast species are sometimes found in the soil of vineyards than in the neighbouring meadows. The cells in the vineyards are dried up and killed, whereas in the meadows where the cells are protected from drying, life is maintained, and the cells multiply. In such places cells also occur during the hot season of the year, and here their propagation goes on most vigorously. Where the ground is subject to drought the variation brought about in the course of years may be altogether extraordinary.

The soil must, therefore, be considered the chief habitat of yeast at every time of the year. They are carried from the earth by means of wind and rain, as well as by the action of insects and other creatures, to the sweet juicy fruits, where they multiply vigorously; a few fall to the earth again, whilst others are carried to secondary places of incubation. When the fruit is ripe the wild yeasts thus strongly developed find their way into the fermentation industry. It is only if they are allowed to remain, to multiply, and to obtain a secure footing, that they are capable of bringing about any disturbance in the industry. Otherwise they are immediately suppressed by the large quantity of the culture yeast added to the nutritive liquid.

During their development on grapes and other juicy fruit the yeast cells compete for nutrition with many other organisms, including bacteria and moulds. These observations led Wortmann to adopt the view that the true importance of alcoholic fermentation is biological. Most of the competitors of yeast can multiply much more rapidly, and would soon suppress it if no means existed for restricting their growth. This means is supplied by the alcohol produced by the yeast cells, whereby they are able to poison their enemies. Wortmann showed how the poisonous action of alcohol is apt to support yeast in competition with other organisms. During the early stages of the development of yeast in must a surface growth of various organisms can be observed. Amongst these the small apiculate yeast is especially prominent, and this soon

brings about a fermentation. The alcohol so formed suppresses most of the moulds. The true wine yeasts now gradually begin to develop, and simultaneously the development of wild yeasts, of bacteria, and of the *Dematioid* species, ceases. As soon as the alcohol content rises above 4 per cent., as a result of the activity of the true yeasts, *S. apiculatus* is suppressed, and the wine yeasts immediately take command of the field to such an extent that, in an ordinary microscopical examination, nothing but their cells can be observed. The most powerful alcohol-formers amongst the yeasts again gradually supersede the weaker species.

Temperature plays a great part in the life of yeast cells, and Hansen has made use of this relationship as one of the most important means for characterising the species.

In 1883 he proved that both the spores and vegetative cells of different species possess different powers of resistance to heating in water. In this respect the spores are more resistant than the vegetative cells.

In such determinations the condition of the cells has a marked influence, and the result depends largely upon their age. Thus the two-day-old cells of *S. ellipsoideus II.* grown in wort at 27° C. were killed on warming to 56° C. for five minutes in sterilised distilled water, whilst cells similarly prepared, but two and a half months old, were heated to 60° C. for five minutes without being destroyed.

Ripe spores of this species, developed at 17°-18° C., and partially dried for eight days at the same temperature, withstood heating for five minutes at 62° C., but not at 66° C.

The vegetative cells of *S. cerevisiae I.* were killed by five minutes' heating at 54° C., and the spores at 62° C.

An interesting classification of Hansen's six species in relation to any given temperature, is obtained by cultivating them in wort under conditions favouring the formation of films. Thus, if the development is carried out at 36°-38° C., the three *Pastorianus* species are killed in eleven days, whilst *S. cerevisiae I.* and the two ellipsoid species remain alive. From this and similar experiments, it may be argued that the rule formerly accepted that top-fermentation yeasts can

develop at a higher temperature than bottom-fermentation yeasts has no general application.

Kayser's more recent work along the same lines has confirmed these results. He also proved that the species withstand considerably greater heat in a dry than in a moist condition. Thus a yeast species isolated from pale ale was killed in a moist condition by heating for five minutes at 60°-65° C., whilst in a dry condition it withstood a temperature of 95°-105° C., and in the case of a wine yeast (St. Emilion), the corresponding temperatures were 55°-60° C. and 105°-110° C. The spores withstand temperatures 10° and even 20° higher than the vegetative cells.

Vegetative cells which are derived from the heated spores show a somewhat greater power of resistance than normal vegetative cells. This increased power of resistance is not transmissible; by cultivation in beer-wort it disappeared entirely in the second generation.

The temperature limits within which budding of cells can take place in wort were investigated by Hansen. The upper limit for *S. Past. I.* is 34° C., for *S. membranæfaciens* 35°-36° C., for *S. anomalus* and *S. Ludwigi* 37°-38° C., for *S. Past. II.*, *III.*, and *S. ell. I.*, *II.*, and for *S. cerev. I.* about 40° C., and for *S. marxianus* 46°-47° C. The lower limit for each of these species is 0.5° C., with the exception of *S. cerev. I.* and *S. Ludwigi* with a limit of 1°-3° C. Müller-Thurgau found that the wine yeasts that he examined are incapable of propagation at temperatures above 40° C.

It is, of course, impossible to establish any one temperature that shall serve as the optimum for the growth of yeast cells, because the composition of the nutritive liquid has a greater effect than it has on other determinations. The formation of new cells in the same liquid goes on at a diminishing rate when the development proceeds at a constant temperature, because the increasing quantity of the products of metabolism and the simultaneous impoverishment of the nutritive fluid acts restrictively upon the growth, especially at higher temperatures. An approximate temperature of 28°-30° C. is found to be favourable for the development of many species. Without doubt the species behave differently in this respect as well as

in regard to the maximum production of yeast which can be developed from a given inoculation.

Many fermentations take place in the industry at lower temperatures; indeed, in the case of bottom-fermentation breweries, very considerably lower than the optimum for the multiplication of the cells. In order that fermentation may be completed at so low a temperature within a reasonable time, and before other organisms have an opportunity of infecting the liquid, relatively large amounts of yeast are introduced, and propagation is assisted by aeration. At times the pitching yeast is first placed in a smaller quantity of the liquor at a higher temperature (about 20° C.), allowed to grow for a few hours, and the newly-formed and vigorous cells are then introduced into the cold liquor. There appears to be a tendency to forego the extremely cold fermentations once customary in many places. In distilleries, where fermentation proceeds at a higher temperature, it is often necessary to take special precautions to avoid a considerable rise in temperature during the first stages of the fermentation; otherwise the propagation of cells ceases too soon. Consequently the growth would be so enfeebled that it would be impossible to carry the fermentation to completion.

It has already been stated that the composition of the nutritive fluid plays an important part in the propagation of the yeast cells. Liquids containing a large percentage of sugar have a weakening effect on the cells. According to Laurent, growth ceases in a decoction of malt germs containing 60 grammes of sugar per 100 c.c. A few species of yeast, nevertheless, appear to retain their activity even in the presence of greater quantities of sugar. The aeration of yeast, as carried out in practice, is of real importance for propagation. Exact conclusions regarding this were published by Hansen in 1879. He used the cell-counting chamber, already alluded to, and found that a beer yeast grown in wort at 12°-14° C. showed the formation of eleven cells from a single cell in sixty hours without aeration, whereas with aeration thirty-six cells were formed from each individual in the same time. The importance of aeration depends not only upon the fact that oxygen reacts more intensely on the indi-

vidual cells, but also upon the removal of the products of metabolism. The stirring up of the cells brought about by air bubbles brings them constantly into contact with fresh portions of the nutritive fluid. In the air-yeast factories this fact is utilised, and a considerably higher yield of yeast is obtained than in the older process (foam yeast). According to Delbrück, it is found in practice that 100 parts of malt yield 21 to 23 parts of pressed yeast in a non-aërated wort, and 30 parts in an aërated wort. He further proved that the higher yield was reached after four and a half hours' aëration.

For brewery yeasts the aëration of the wort is of special importance, as the clarification is dependent upon it. \*In a badly-aërated wort the yeast does not readily settle out. This circumstance must not be neglected even during the growth of the pure culture in the flasks.

Amongst the products of metabolism removed by aëration, carbon dioxide deserves special mention, for it exercises a restrictive effect on the multiplication of the yeast. This has been proved by parallel experiments in open and closed vessels under conditions otherwise identical. If, however, a comparison is made between the amount of alcohol formed in the two vessels and the quantity of yeast produced, it will be found that the individual cells in the closed vessel have produced a larger quantity of alcohol than those in the open vessel. The carbon dioxide appears, therefore, to exercise a favourable influence on the fermentative power of yeast.

The action of light on yeast cells is described in the general review of the physiological properties of fungi.

#### Variations in the *Saccharomyces*.

Hansen's numerous investigations proved that the *Saccharomyces* are affected in varying degree by external agents, and that it is possible by suitable treatment to bring about variations along different lines. Even the individual peculiarities of cells in a pure culture may be of importance in this respect. Some of these changes are only evanescent. By suitable cultivation they disappear, and the species returns to its original condition. Others are more deeply seated, and

it is only by a special treatment that the culture can be deprived of its newly-acquired properties. In certain cases, it is found impossible, even after years of methodical treatment, to cause a growth to revert to its original state.

1. The times given for the appearance of the first indication of spores are based upon the understanding that the growth has been cultivated at 25° C. for twenty-four hours in wort. In 1883 when Hansen published temperature curves for his six species, he found that growths which had been developed for two days instead of one, at the same temperature, developed spores more slowly and less freely than usual. If, however, they are subsequently treated in wort in the way described, the normal conditions are re-established. This forms an example of a very feebly-rooted variation.

2. In a gelatine culture, Carlsberg bottom yeast No. 1 is often found in both oval and elongated sausage-shaped cells. If a colony derived from each of the cell forms is transferred to flasks containing wort, a growth is again obtained consisting partly of oval and partly of elongated cells. Hansen's experiments proved that the latter when cultivated in new flasks retained to some extent the sausage-like form, and when transferred to the pure culture apparatus the growth continued to show a mixture of such cells, but when the yeast was conveyed to an ordinary fermenting tun they disappeared. The variation in this case is, therefore, a more deeply seated one. It only ceases when the yeast has been transferred through a series of fermentations. Another example is shown by a bottom yeast which, after a long period of stunted growth, had been propagated in wort at about 27° C., and formed cells with a normal appearance, whilst the growth cultivated at 7 $\frac{1}{2}$ ° C. gave entangled colonies with mycelial branchings. This forms a striking example of the effect that temperature has upon the form of cells.

3. Hansen's observations of *S. Ludwigi* supply an illustration of a far-reaching change in the character of the cells. If single individuals are grown as pure cultures, growths are obtained which show a marked difference in their power of spore-formation. By systematic selection of single cells, Hansen succeeded in producing growths which gave no spores

under the usual conditions, and conversely, it was possible to select a yeast colony derived from a cell containing spores, and by further cultivating the colony to obtain a growth which possessed the power of freely generating spores. By such systematic choice the species was divided into three forms—one distinguished by its vigorous spore-formation, another by the fact that this power had almost disappeared, and a third, which could not form spores. By frequent infections in wort the third form reverted to the power of forming spores. This took place slowly, but when Hansen transferred it to a 10 per cent. dextrose solution with yeast decoction this property was instantly restored.

In other species, varieties which have lost their power of spore-formation completely, or in part, may make their appearance, without any known cause, both in liquid and on solid nutrient media. In some cases (e.g., *S. Ludwigi*) that power is restored if dextrose is added to the nutrient liquid. Similar observations regarding asporogenesis have been recently made by Beijerinck on *S. octosporus*.

If a pure culture of brewery yeast is developed in a wort which has not been aerated after sterilisation, it generally loses its normal "breaking" and clarifying properties, under brewery conditions, and this to a degree dependent on the species. These new variations must often be cultivated through a great many generations in ordinary brewery wort before regaining the original qualities of the species. As aeration brings about changes in the chemical composition of the wort, it is evident that the effect on the protoplasm is due to such circumstances.

The author of this book showed in 1890 that when a brewery top-fermentation yeast which has given a good clarification in practice is kept for some time in wort-gelatine at room temperatures, it tends to lose its clarifying properties for a considerable time. At the same time, it brings about a considerably stronger attenuation than in its original condition.

As an additional instance of the effect of the chemical composition of wort in producing new varieties, we may mention the observation, due to Hansen, that *S. Pastorianus I.*, which imparts an unpleasant taste and smell to beer-

wort, is apt to lose this power for a time if preserved in an aqueous solution of cane sugar.

A similar proof of a variation in brewers' low-fermentation yeast, due to the composition of the nutrient liquid, was furnished by Seyffert, who found in the case of a selected type which, after long use in breweries, had lost its good properties with regard to clarification, that it was possible to restore it to its original condition by treatment with lime. Gypsum was added either to the wort, the brewery water, or the steeping vat, and from wort prepared in this way wort-gelatine was concocted, in which the degenerated yeast growth was sown for fresh pure-cultivation. On development of the colonies in small flasks, these new growths showed true "breaking" and the power of adhering to the bottom of the flask; the qualities thus regained were retained during the use of this yeast in practice.

Another example of physiological transformation is the following:—The three species described by Hansen under the name *Saccharomyces Pastorianus* form a dough-like sediment under certain conditions similar to those of the other *Saccharomyces*; under other conditions, however, a film-like, wrinkled, or caseous sediment consisting of small lumps (Pasteur's *levure caséuse*)—a sediment of very different appearance. In the latter case, the fermenting wort also assumes a characteristic appearance, and, contrary to what ordinarily occurs, remains bright throughout the fermentation, so that yeast flakes may be observed rising to the surface and sinking again to the bottom. If this curious sedimentary yeast is repeatedly cultivated by new fermentations in wort, it can be again transformed into the dough-like condition.

Both Hansen and the author established the fact that by long storage under ice, and subsequent growth in wort, a brewery bottom-fermentation yeast exhibited top-fermentation phenomena, which, however, by continued pitching gradually but entirely disappeared. Similar observations have been made by Will.

We also find a transitory physiological transformation in film-formations of the *Saccharomyces*.

4. In 1889, Hansen published the results of a series of

experiments which were undertaken with the hope of discovering the conditions causing variation, and of experimentally bringing about the formation of new races, and if possible new species. He has since published additional work on the subject.

(a) He found in the case of typical *Saccharomyces* that when their cells were cultivated in aërated wort\* at a temperature above the maximum for their spore-formation, and near the maximum for their vegetative growth, they were affected in such a manner that they lost their power of forming spores and films (*Asporogenesis*). This was also true of the innumerable generations successively formed in new cultures under the most varied conditions. The starting point was always a growth which showed not the slightest trace of asporogenous cells. For example, it may be noted that *S. Past. I.* loses its power of forming spores by treatment at 32° C. In the case of the wine yeast, *Johannisberg II.*, this occurs at 36° C. In the seventh culture of *S. Past. I.* all the cells were asporogenous. Hansen succeeded also in bringing about a transformation by cultivation on solid media. Such asporogenous growths were formed in the case of *S. Past. I.* on wort-gelatine at 32° C., when inoculations were made at shorter intervals, as is commonly the case when liquids are used.

In some of the species treated in this way, it was also observed that they yielded a more abundant crop of yeast in wort-cultures, but a slower fermentation. This was, for instance, the case with Carlsberg low-fermentation yeast No. 2. The newly-formed variety attenuated more slowly and weakly than the original species; but at the same time the clarification was better.

Rayman and Kruis have shown that the cells present in films possess the power of oxidising alcohol produced during fermentation, into carbon dioxide and water. Hansen's varieties, while completely losing the power of forming films, are rendered incapable of performing this oxidising action. Thus, while a flask, containing the original species, which had developed a luxuriant film after six months' standing, showed only 1.5 per cent. by volume of alcohol, a parallel flask, which

\* By repeated shaking of the successive cultivations.

showed no film-formation, contained 5.5 per cent. of alcohol—a quantity equal to that found at the end of the first month.

In another series of experiments Hansen showed that the action of higher temperatures upon the cells without aeration was capable of producing radical and lasting alterations of a different kind in the nature of the protoplasm. When Carlsberg yeast No. 1 was cultivated in wort at 32° C. through eight cultures, each successive culture being inoculated from the preceding one, which had been left undisturbed until the end of the fermentation, a variety was evolved in the ninth culture which produced 1 to 2 per cent. by volume less alcohol than the original form, in wort of 14° Balling, containing 10 per cent. of saccharose. The new variety clarified better under brewery conditions, and gave a weaker attenuation at the end of the primary fermentation; a similar behaviour was noted in the case of other species.

(b) Hansen also succeeded, by cultivation in nutrient gelatine, in producing new stable varieties.

Thus, two varieties of Carlsberg low-fermentation yeast No. 1, each generation of which was transferred to the surface of wort-gelatine, attained a fermentative power superior to that of the original forms. The difference is still more marked when cultures are developed from spores of the top-fermentation yeast *S. cerevisiae* I. on yeast-water gelatine. The new varieties produced 3 per cent. more alcohol than the parent form.

The observations already detailed regarding asporogenesis lead to the interesting conclusion that a species can lose one of its characteristic properties as a result of external influence, and that virtually a new species is produced.

In the course of Hansen's experiments on spore transformations brought about by the action of temperature and aeration, it was observed that if cells of successive generations were removed many were affected even in the first growths under the new conditions; this modification, however, is temporary in character; it is only after successive generations have been allowed to develop through continued inoculation under the new conditions that the acquired characters become

constant. It appears from this that the transformation does not depend on temperature or aeration alone, but also on the nutrition and propagation of the cells.

A comparison of these different factors has, however, shown that they contribute unequally to the result. Both the nutrient liquid and the aeration are only of importance in bringing about vigorous new formations, and may, therefore, vary considerably in strength without materially affecting the result. This, however, is not true of temperature; a fluctuation of a few degrees is sufficient to prevent the variations described from coming into existence. Hence, it follows, that temperature plays the principal part in these transformations.

As previously stated, these remarkable changes are only brought about by a long-continued and violent interference with the vital processes of the cells; they do not occur so long as development takes place in the normal manner.

An example of the way in which the *Saccharomyces* cells retain their power of forming spores under ordinary conditions is supplied in breweries and distilleries. Here culture yeasts have existed continuously for centuries, and untold generations have been produced under conditions which would not allow, as a rule, of this function being brought into play, and yet the power remains intact.

Lepeschkin observed a well-developed mycelium formation in *Schizo-saccharomyces Pombe* and *mellacei*, which he regarded as a stable variation brought about by the alteration (mutation) of certain cells.

Hansen observed a remarkable variation when young growths of *S. ellips.* II. and the wine yeast *Johannisberg* II. were preserved for a few months in Freudenberg flasks in shallow layers of wort at 0.5° C. A few cells of these bottom-fermentation yeasts gave top-fermentation phenomena. The further investigation showed that selection had taken place. The top-fermentation yeast cells remained continuously as top yeasts, the bottom-fermentation yeast cells as bottom yeasts. A similar state of things was observed during the examination of a large number of cells from old cultures of brewery bottom-fermentation yeasts. On the other hand, similar cultures both of wild top-fermentation and of brewery

top-fermentation yeasts yielded only a small number of cells which displayed bottom-fermentation phenomena. We are not dealing, therefore, as in the previous cases (asporogenesis) with the action of definite factors producing a transformation, but with unknown causes, and probably with sudden variations of the same kind as the mutations studied by H. de Vries. According to these researches the two physiological forms, top- and bottom-fermentation yeasts, are not independent. On the contrary, they may both occur in a growth derived from an individual cell. They can exist together in the same liquid, one or other securing the upper hand in their competition and thus determining the character of the growth.

Since 1887 the author, who has long enjoyed the co-operation of his laboratory superintendent, H. Rafn, has treated as one of his principal problems the study of the variations of yeasts during their application in the different branches of the fermentation industry. The number of his investigations has now increased to many thousands. The difficulty in work of this character, where large masses of yeast are under investigation, is to make sure that the growths observed by the separation of a certain number of cells with abnormal characteristics are real varieties of the parent cells, and have not been derived from infection by foreign species.

The botanical and biological investigation can never form more than part of the examination, and must, moreover, be carried out with the utmost care. We must take refuge to a great extent in the different characteristics that are developed, partly during large scale fermentations and partly during parallel fermentations carried out in the laboratory with small quantities. For such experiments it is obvious that only yeast masses can be used which have been derived from a single cell.

As a result of observations carried out during a series of years, it has been definitely established that variations do very frequently take place. They occur without any obvious cause, and on occasion they may develop in such quantities that the whole mass of yeast changes its character or "degenerates." This expression, which is used in practice, only indicates that the yeast mass in the special brewery or distillery concerned no longer suffices for the particular requirements.

It does not indicate what the true value of the yeast may be for this branch of the industry. Thus occasionally such a yeast mass that has altered its character has produced excellent results when applied in other places where the requirements are different. A selection of a cell from the yeast mass that has not degenerated has often proved the basis for regeneration, in that the new culture possesses the properties of the original stock.

A very cautious treatment of a sample of purely cultivated yeast will throw some light upon this question. If a number of cells are separated from a yeast mass derived from a single cell, which has been in use in the industry for some time, the pure cultures from these cells will show differences in a set of parallel fermentations, and sometimes important differences in respect to taste, smell, and other characteristics of the fermented liquid ; also as regards the attenuation, the character of the yeast layer, etc. Varieties may, for instance, occur which produce a penetrating and unpleasant bitter flavour, but in every other respect give a result in agreement with the culture yeast. Thus it is interesting to record a case where a selected variety gave considerably more rapid clearing than the original race, whilst in every other respect, practical and biological, it was identical. In other cases the power of attenuation varied greatly. By studying a number of selected growths a series of intermediate forms could be detected, and by a proper selection cultures were prepared which gave the normal attenuation in wort of the same character.

A problem of great practical and theoretical importance is to decide if such variations occurring in the yeast mass in practice are constant or of a purely transitory nature. Hansen adopted the view that, as a rule, "the races prepared from industrial yeast cannot be maintained, but disappear," and that "so long as the beer yeasts are kept under brewery conditions, they only display slight alterations, which are of a transitory character." This view, however, is in contradiction to the results repeatedly obtained in the author's laboratory. Strongly marked abnormalities may occur in practice in the work of single cells, and certain of these variations prove to

be of a stable character both when applied on the large scale, and also when stored for years in a 10 per cent. cane-sugar solution. There are variations still kept in the laboratory which after preservation in such a solution for more than ten years still retain their properties. These races, therefore, do not disappear.

It follows that in the preparation of pure cultures in yeast to be applied in a brewery, a distillery, a wine fermentation, etc., we cannot reckon on dealing simply with a type ready to hand in a pure condition, but rather with a mixture of elements, often of a highly different character, even if the mass of yeast has been originally derived from a single cell. By the process of pure cultivation based upon a detailed knowledge of the special practical requirements, a form can be prepared of the required type. Such work can never be attempted at random, but must consist of systematic research carried out with rigid rules. How long such a type may be preserved in practice before it develops such pronounced varieties and in such quantities that the character of the yeast mass experiences a change, depends to a great extent upon circumstances which are still unknown.

It will be seen from all this that the principle applied in the author's laboratory in carrying out the pure culture of brewery, distillery, and wine yeasts, etc., is based on a reliable starting point, and the experience gained during the long time that has elapsed since the laboratory was instituted has only served to confirm the correctness of the author's view.

The improvement of yeast, about which the author has published his views, consists in selecting cells taken from a mass of yeast which has given satisfactory results, and preparing growths which display the desired characters in greatest perfection. This treatment is carried on through several generations, and in each case after the mass of yeast has been applied for some time in practice.

These observations have no connection with any variation in the composition of a nutritive fluid. They are simply concerned with comparative experiments with selected and absolutely pure cultures.

### Morphology and Anatomy of Yeast Cells.

**Yeast Deposits.**—Hansen's investigations in 1881-1883, which took the form of a direct study of the growth of a single cell under the microscope, and of growths derived from a single cell, made it possible for the first time to give exact descriptions of the different species of yeast. He proved that the shape, relative size, and appearance of the cell are not sufficient in themselves to characterise a given species, for the same species may exist in different forms under differing external influences. At the same time he established the fact that the shape may provide valuable indications, as the various species may react in a different way and with a different shape when the same influence is brought to bear.

As an example of the results which may be obtained by a comparison of young deposits of yeast, the six varieties isolated by Hansen may be quoted (*S. cerevisiae* I., *S. Pastorianus* I., II., III., *S. ellipsoideus* I., II.).

The growths are developed in the following manner:—The cells, after short cultivation in wort, are introduced into fresh wort, and brought to vigorous development at 25° to 27° C. in twenty-four hours. If then *S. cerevisiae* I. is compared with the three *S. Pastorianus* species, the general appearance is strikingly different. *S. cerevisiae* I. consists predominantly of large round or oval cells, and *S. Pastorianus* chiefly of elongated sausage-shaped cells, but it is a very different matter if the cells of the first are mixed with cells of one of the second species. It then proves to be impossible, by simply noting the form, to distinguish between the larger and smaller oval and roundish cells of *Pastorianus* and many of the *cerevisiae* cells. The two species, *S. ellipsoideus* I. and II., are predominantly oval and round. Sausage-shaped cells occasionally occur, and here again it is impossible, simply by studying the form, to determine the species when *S. cerevisiae* or *S. Pastorianus* are mixed with them.

By direct measurement of the sedimentary forms it is also impossible to discriminate them.

On examining pictures of these six pure cultures, it will be seen that we are dealing with three different divisions of

budding fungi, one of which is represented by *S. cerevisiae* I., the second by the three *Pastorianus* species, and the third by both the *ellipsoideus* species. So much and no more can be established by a purely microscopical observation, and this only under the particular culture conditions described.

The development of the yeast cell takes place through **budding**, a slight swelling appearing in the mother cell, which increases in size. According to Kny, budding follows with equal rapidity both in light and darkness. As soon as the new cell has attained a certain size it can form a new bud, and this process of budding continues until a group of budding cells is formed. The cells may break away from each other at an earlier or later stage, so that the group may consist of a varying number of individuals. The development of the yeast cell was observed by Mitscherlich in 1843. The daughter cell may assume a totally different form from the mother cell. This may also take place in the industrial species, including those which give fairly uniform oval cells in the large fermenting vats. For example, ordinary brewery, low-fermentation yeast may, for reasons unknown, produce cells with the appearance of *Pastorianus* and *ellipsoideus*, so that it is impossible, under the microscope, to distinguish whether such a culture yeast is infected with a foreign yeast or not.

As an example of the change of form brought about by an unknown cause in the case of industrial yeast, it may be mentioned that, by excessive treatment with air, the air-yeast of the pressed-yeast factory alters from an oval or elliptical to a much elongated *Pastorianus* shape.

In general, it may be stated that low-fermentation yeasts form groups containing fewer cells than is the case with top-fermentation yeasts. There are, however, many exceptions to this rule. It is impossible to indicate any universal type of microscopical picture for the two groups of yeasts, and the same holds good for the general picture of a single race of culture yeast. It is only by exactly comparable growths carried out in parallel experiments in the laboratory that it is possible to establish differences between the general appearance of the races. When applied in practice, so many different factors come into play that the appearance of the growth may

entirely alter its character. On these lines no starting point can be found for an analytical examination of yeast to determine its purity.

A peculiar group of yeasts, the *Schizo-saccharomycetes*, are distinguished from others by the formation of daughter cells through division of the mother cell, a cross-section being formed in the latter.

**Film Formation.**—It is well known that fermenting and fermented liquids are covered with film growths. It was first shown with certainty that *Saccharomyces* (in the strict sense) are able to form films by Hansen's observations on cultures derived from single cells.

The universally occurring *Mycoderma* species form films easily and rapidly. Some also give fermentation phenomena; others do not. Such a film is greyish on beer and wort, with a dry appearance, and in its later stages wrinkled and lighter in colour. Amongst the cells there is a considerable admixture with air. Similar films are formed by a few of the *Torula* cells. The film of *Chalara Mycoderma* is gelatinous, and has a bright appearance. In the case of *Monilia*, which may occur with budding cells, the film formation is peculiar. During the vigorous fermentation, a film forms on the froth, which gradually spreads over the whole surface, and is occasionally wrinkled. The cells in the flask form a deposit, produce a vigorous fermentation, and rise with bubbles of carbon dioxide to the surface again, where they begin a new stage of development. If sterilised lager beer is inoculated with this fungus, no fermentation takes place, and a thin dusty film is formed, but under other circumstances the fungus forms white, floury, and woolly layers like *Oidium*.

The films of true *Saccharomyces* differ somewhat from these. As a rule, they are produced in the following way:—If cultures are allowed to stand undisturbed for a longer or shorter period in wort at room temperature, it will be found that small specks of yeast appear on the surface of the liquid at the completion of the primary fermentation. These collect together at a later stage to form islands of varying size and shape, with a flat upper and arched lower surface. Finally these fuse together to form a light greyish-yellow and slimy film,

which often spreads up the wall of the vessel forming a complete ring. Such a complete film-formation only takes place when the primary fermentation is completed. If the flask is shaken, shreds of the skin are loosened and sink, and in this way a complete layer may be collected on the bottom, whilst the skin reforms and assumes a mottled appearance, the younger portions being thin and dark, whilst the older are thick and pale in colour.

The necessary condition to enable the film to form is the presence of a free and undisturbed surface with access of air. A vigorous film-formation assumes a free access of air. The function of film-formation is subject to the same conditions as the formation of endospores.

Along with film-formation a bleaching of the wort takes place, which now assumes a light yellow colour. This occurs more rapidly at a high temperature, and is most readily observed in those species which bring about the most vigorous film-formation. Erlenmeyer flasks half-filled with wort and covered with filter paper are admirably adapted for such cultures. A few drops of a young and vigorous growth of yeast should be introduced.

Hansen undertook the following determinations :—

- (1) The temperature limit for the formation of films.
- (2) The approximate time required for the first appearance of the film at different temperatures.
- (3) The microscopical appearance of the growth at different temperatures.

The main object of comparative observations of this kind lies in determining the microscopical appearance of films at similar temperatures.

The examination of the film was undertaken when it had just developed sufficiently to be visible to the naked eye.

A glance at the illustrations representing these film-growths (see description of species) will show that their general character differs from that of the sedimentary forms. For instance, the sedimentary form of *S. cerevisiae* *I.* is oval or spherical, whilst in the film, elongated and mycelial cells quickly appear, and the growth gradually assumes an appearance quite distinct from that of sedimentary yeast.

If we compare the film-formation of the six species, we find that the films developed at the higher temperatures offer very little scope for discrimination, *S. cerevisiae I.* and *S. ellipsoideus II.* alone being distinguishable from the remainder. It is quite otherwise, however, when young films developed at 13°-15° C. are examined. The two species, *S. Pastorianus II.* and *S. Pastorianus III.*—both top-fermentation yeasts, the cells of which in ordinary cultures cannot be distinguished from each other with certainty—exhibit in this case entirely different forms of growth. An equally striking difference is found between the otherwise similar species, *S. ellipsoideus I.* and *II.*

Observations of the limits of temperature for the formation of films show that for *S. cerevisiae I.* and *S. ellipsoideus I.* these lie approximately within 38° and 5°-6° C.; the limits for the three *Pastorianus* species are 34° and 3° C.; *S. ellipsoideus II.* has the same lower limit as the last species, but its maximum temperature is 38°-40° C.

The time limits, compared with those given for ascospore-formation, show that in both cases development takes place more slowly at low than at high temperatures.

At temperatures above 13° C. the film of *S. ellipsoideus II.* develops so rapidly and vigorously that flasks containing this yeast can be recognised by this alone. Thus, at 22°-23° C. the film had completely covered the surface in six to twelve days, whilst the other five species required three times as long to form a film, and this was generally more feebly developed. This species and *S. Pastorianus III.* also develop a vigorous film with comparative rapidity at the ordinary room temperature, the other species being left far behind.

A further important biological relationship is the following:—Hansen's investigations have proved that the temperature maximum for budding in wort is higher than the maximum for film-formation, and that this again is higher than the maximum for spore-formation; in other words, with a rising temperature, a point is reached at which spore-formation ceases, then a higher point at which film-formation ceases, and lastly, a still higher at which budding is no longer possible. On the other hand, the experiments indicate that the tem-

perature minimum for film-formation is lower than that of spore-formation.

In brewers' low-fermentation yeasts, and in some wild yeasts, Will observed round and oval cells, having a thick membrane and containing a number of small oil-drops (Fig. 48). These occurred in the rings of yeast and in the small surface patches preceding true film-formation. If treated with concentrated hydrochloric acid, the membrane splits into two

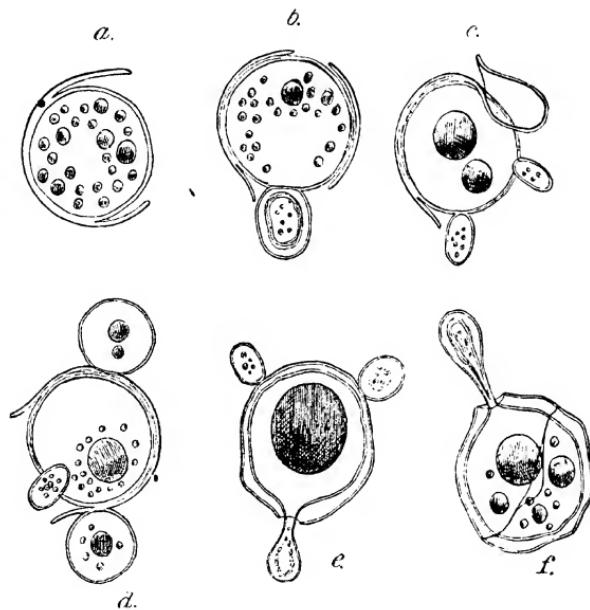


Fig. 48.—Resting cells (after Will).—The outer layer is partly or completely detached.  
*a, b*, in wort; *c, f*, in mineral nutrient solution.

layers. In cultures, especially in artificial nutrient liquids, the outer layer of this membrane gradually detaches itself; sometimes in such a way that the outer layer is not torn, so that it appears as though the one cell were contained within the other. The cell contents are coloured green or brown by concentrated sulphuric acid. The glycogen reaction with iodine has been occasionally observed in the cells. They appear to play a certain part in the life economy of the growth,

as resting cells, for these cells are sometimes found alive in old growths when most of the other individuals have perished. In artificial nutrient solutions containing mineral salts, sugar,

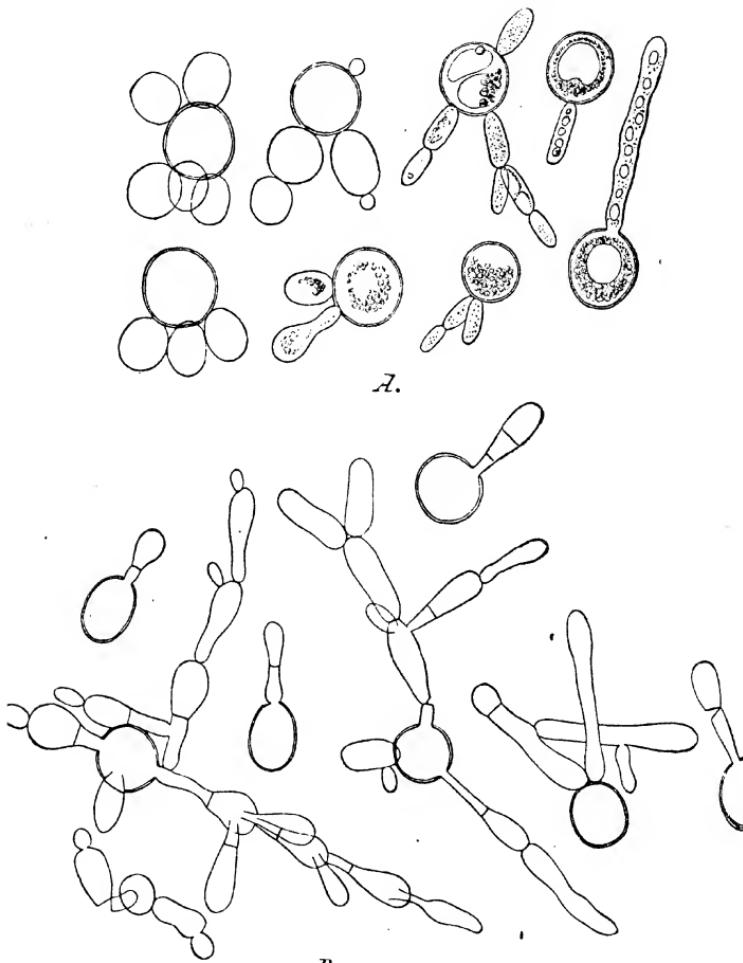


Fig. 40.—Resting cells (after Will).—A, usual mode of germination; B, resting cells with club or sausage-shaped daughter cells with transverse walls.

and asparagin, with addition of citric or tartaric acid, such resting cells occur also in the sediment. Globular or oblong

yeast-cells germinate from the resting cells, either singly or in large number (Fig. 49, A). Club-shaped cells with transverse-wall formation frequently arise, especially in older cultures of resting cells produced in mineral nutrient solution. This phenomenon may recur in derived growths (Fig. 49, B). During germination on a solid nutrient medium, Will also observed a splitting up of these transverse walls (Fig. 49, B).

According to Rayman and Kruis the cells of the film have a marked respiratory power, oxidising the alcohol formed into carbon dioxide and water, and at the same time splitting up the albuminoids of the liquid into amides and ammonium salts of organic acids.

**Cultures on Solid Substrata.**—After Schroeter and Koch had shown that by cultivation on solid media, species of bacteria display distinct characteristics, Hansen succeeded in proving that a similar relationship holds good for yeasts. For this purpose he utilised beer-wort, to which about 5.5 per cent. of gelatine had been added, contained in flasks closed by means of cotton-wool plugs. When these flasks are inoculated with the six species (*S. cerevisiae* I., *S. Pastorianus* I., II., III., *S. ellipsoideus* I., II.), and allowed to stand at a temperature of 25° C., the growths which develop (streak-cultures) show such macroscopic differences in the course of eleven to fourteen days that four groups may be more or less sharply distinguished. *S. ellipsoideus* I. stands alone, for its growth exhibits a characteristic net-like structure on the surface, which enables it to be distinguished from the other five by the unaided eye. When gelatine with yeast-water is employed for such cultures and the experiments conducted at 15° C., *S. Pastorianus* II. yields growths after the lapse of sixteen days, the edges of which are comparatively smooth, whilst the growths obtained from *S. Pastorianus* III. are distinctly hairy. A microscopical examination shows that the two species are also distinguishable morphologically. This is by no means always the case with cultures on solid media; in fact, the differences are often less marked under such conditions than when nutritive liquids are employed.

For the *Mycoderma* species and *S. membranæfaciens*, Hansen discovered a characteristic behaviour in wort-gelatine in which

they form shield-like colonies readily distinguishable from those of the *Saccharomyces*.

In this connection we may mention Hansen's observation that some species—*e.g.*, *S. Marxianus* and *S. Ludwigii*—can develop a mycelium when grown on a solid medium, while others are unable to do so.

The characters which can be obtained in this way fluctuate greatly, for both the behaviour of the living material and of the substratum may be strongly modified by other external influences. This fact was brought out by Will in a special study of four species of bottom-fermentation beer-yeasts. He discovered that when the inoculating material contained cells from the film they exercised considerable influence on the appearance of the colony.

The giant colonies investigated by P. Lindner were prepared by transferring a drop-culture containing a vast number of cells to a spot on the nutritive gelatine. It gradually develops a large rounded colony which can be photographed. Even under these conditions the picture fluctuates for one and the same species, according to the differing circumstances. In certain cases, by the use of such growths, strongly marked differences can be produced, as is the case with the usual plate and streak cultures. Frequently, however, the differences between these giant colonies are so minute that it is impossible adequately to describe them.

Aderhold, during an examination of gelatine-growths of German ellipsoid wine yeasts, found that in puncture-cultures and giant-cultures two types were distinguishable, one of which showed colonies with funnel-shaped depressions and with marked concentric lines, whilst the other showed conical growths with indistinct concentric structure, but very prominent radial streaks.

A great number of yeast species liquefy nutrient gelatine. This was proved by the author in 1890 with respect to brewers' high-fermentation yeasts. Subsequently Will, Wehmer, and others made the same observations with other yeasts.

**Structure and Character of Yeast Cells.**—During the growth of the cell the membrane gradually becomes more distinct.

When the cell is fully grown the strength of the membrane depends on the concentration of the nutritive fluid. It has a tendency to thicken in liquids with a high percentage of extract; especially marked thickening is met with in the resting cells occurring in films. By treatment with concentrated hydrochloric acid a division of the cell wall can often be observed.

The gelatinous network first observed by Hansen may be regarded as a special development of the membrane, reminiscent of the zoogloea formation of bacteria. Under certain conditions, which have not yet been defined, the colonies brought about by the budding of yeast cells may combine to form irregular clots which sink more rapidly than individual cells ("break" and clarification in the brewery). This doubtless stands in relationship to a feature of the development of the yeast cell discovered by Hansen in 1884. He found that both *Saccharomyces* and other budding fungi may secrete a gelatinous network which may take the form of strands or plates in which the cells are embedded (Fig. 50, *A, B*). If, for example, some thick brewery yeast is placed in a glass and allowed to remain under cover in such a way that it slowly dries, and then a trace of this yeast is mixed in a drop of water, the network can be clearly seen (Fig. 50, *A*). The formation also occurs in the gypsum block and gelatine cultures. The author has frequently observed this formation in the yeast samples despatched to his laboratory in filter paper enclosed in envelopes.\* Hansen also found it in the film-formations of nearly all species. An ordinary microscopic examination of the pitching yeast in a brewery does not show this fermentation; with the help of staining, however, its presence can be readily detected (Fig. 50, *B*). When the yeast is repeatedly washed, it is no longer possible to detect the network by staining; but if the water is removed, and the yeast set aside for a time and then suitably treated, the gelatinous masses can be readily seen. By varying

\* This method of preserving a sample of yeast is very convenient. A small piece of filter paper is rapidly passed through a flame several times, and a few drops of yeast are poured on to it; it is then folded up, and afterwards wrapped in several layers of paper which have been similarly treated.

the conditions of nourishment of the cells, the development can be promoted or retarded, and the chemical composition modified. The whole behaviour suggests the zoogloea formation of bacteria.

The chemical nature of the wall of the yeast cell is unknown. It is soluble in concentrated sulphuric acid and in concentrated chromic acid. It swells up and becomes transparent in potash and soda solutions.

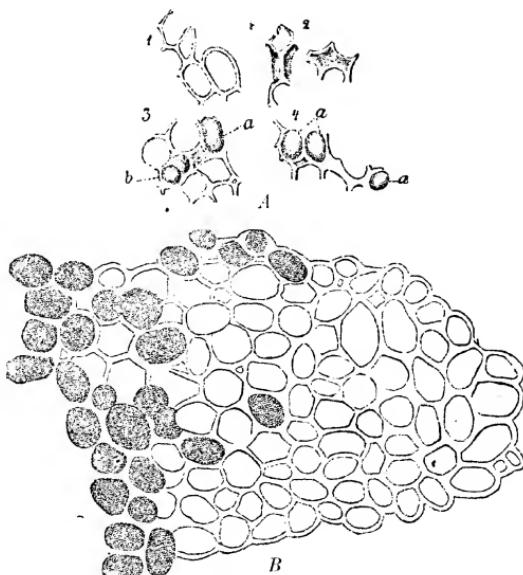


Fig. 50.—Yeast cell with gelatinous network (after Hansen).—*A*, Network obtained by partial drying: 1, portion formed of threads from which the cells have become detached. 2 and 3 show that the network can also form complete walls, such a formation is seen between *a* and *b*—*a* is a negative cell, *b* is a cell with two spores; 4 shows three cells, *a*, embedded in the network. *B*, network with yeast cells, the latter stained by methyl violet, network is not stained. Some of the yeast cells are still in the meshes, but most have detached themselves.

The most important part of the contents of the yeast cell is the cell-nucleus, which is not visible in a direct microscopical examination. It can, however, be detected by a suitable micro-chemical treatment of the cell. (The process for its detection is given in Chap. i.) As early as 1879 F. Schmitz detected a body in the cell by staining, which was undoubtedly the cell-nucleus. His observations were confirmed and

developed by Hansen and Strassburger, and later by Dangeard, Janssens, Wager, Buscalioni, Hoffmeister, Rayman, Kruis, and Guilliermond. We owe much to the fundamental studies and the beautiful and careful drawings of Guilliermond. The nucleus has a rounded form, and appears to enclose a still smaller nucleus. When the yeast cell begins to bud or to form spores, the cell nucleus propagates by division (Fig. 51), and this division takes place before the budding or formation of spores becomes visible. In connection with the fusion of spores observed by Hansen in *S. Ludwigii*, Guilliermond proved that a fusion of the nuclei takes place. He also observed this phenomenon in the case of *S. octosporus*.

The vacuoles constitute another essential part of the cells. They separate gradually from the protoplasm of the young cells, and appear in increasing numbers as pale, feebly-refractive specks. In the older cells they are sharply defined, and may assume highly irregular forms. In the *Mycoderma*

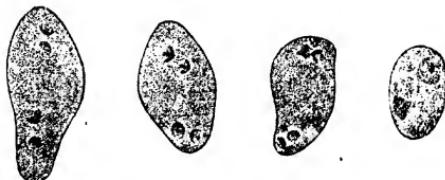


Fig. 51.—*S. Ludwigii*.—Nucleus division during spore-formation (after Guilliermond).

species one or two very large vacuoles are usually found, and this applies also to the old *Saccharomyces* cells. The vacuoles are filled with an aqueous liquid, and often enclose fine granules.

The yeast cells also contain larger and smaller particles of different refractivity, both in the protoplasm and in the vacuoles, which are classed together under the name of granules. They are produced even in quite young cells. At a later stage, when the cells are filled with glycogen, they are not so obvious, but they are sharply defined when the glycogen has disappeared, and they may assume large proportions in the dying cells. Amongst those who have specially investigated the granules Eisenschitz, Raum, Zimmermann, Will, and Guilliermond may be mentioned. In many of these bodies, which doubtless vary in composition, oily or fatty substance have been detected,

as well as albuminoids. Guilliermond showed that by fixing with alcohol and staining with hæmatoxylin or with methylene blue, some of the granules assume a red colour, whilst the protoplasm is coloured blue. As to their importance to the life processes of the cells, Guilliermond and others at present assume that they are chiefly of service as reserve material. Glycogen also constitutes an important constituent of the cell contents. It has already been stated that it is assimilated by the cell when it has a rich supply of available carbohydrates. Its presence can be distinguished by a reddish-brown coloration with iodine in potassium iodide, whereas the albuminoid substances of the cell assume a yellowish colour. On heating the cell, the brown colour disappears, but reappears on cooling. In the fifth section of this chapter it has been shown that glycogen plays an important part in the auto-fermentation of yeast.

**Ascospore Formation.**—In 1839 Schwann discovered that yeast cells can form new cells in their interior, and that these are liberated by the bursting of the wall of the mother-cell. De Seynes gave a clear description of spores in 1868, and in 1870 Reess proved that they are produced by yeast cells of different shape, and that the germination of spores takes place by budding. In 1872 Engel indicated moist gypsum blocks as a specially favourable substratum for the development of spores. Reess, who did not work with pure cultures, regarded these spore-forming yeasts as a special group, which he indicated by the name *Saccharomyces*, a name proposed by Meyen, but he included along with these a large number of species in which no endogenous spore-formation had been observed. Similar conclusions were published by de Bary in his celebrated work *Vergleichende Morphologie und Biologie der Pilze* (1884), which also contains admirable observations regarding yeast fungi.

In 1882-3, Hansen undertook the first experimental investigations concerning spore-formation, and his work made it possible to establish a sharp limit to the group of *Saccharomyces*. The results of his investigations concerning the necessary conditions for spore-formation may be shortly stated as follows:—

1. The cells must be placed on a moist surface and have a plentiful supply of air.

2. Young and vigorous cells can exercise this function most easily and rapidly. Old cells which lack nutritive material can only develop spores with free access of oxygen.

3. The optimum temperature for most of the species yet examined is about 25° C. This temperature favours spore-formation in all known species.

4. A few *Saccharomycetes* likewise form spores when they are present in fermenting nutrient fluids.

A lack of food cannot, as Klebs assumes, be regarded as a direct condition for spore-formation, since young and well-nourished cells can also be induced to form spores immediately—without previous budding—when they are placed under conditions which favour spore-formation, but are unfavourable to budding—*e.g.*, in water saturated with gypsum, but with access of air and at a favourable temperature.

A growth of yeast is developed in the way described on p. 290. Older cultures, developed in saccharine solution or in wort, must be cultivated several times in aerated wort before showing a normal formation of spores. A small quantity is transferred to a previously sterilised gypsum block; this block takes the shape of a truncated cone; it is enclosed in a flat glass dish covered by a larger inverted dish, and is kept moist by half-filling the dish with water.\* If it is desired merely to bring about the formation of spores, the apparatus may be allowed to remain at the ordinary room temperature.

The transferred cells develop through a few generations by means of budding, and then spore-formation begins in the mother-cells.

Hansen was the first to give an accurate description of the structure of spores and a detailed account of their evolution founded upon observations of individuals. He distinguished three typically different groups of *Saccharomycetes* which are

\* *Ascospores* can also be obtained when yeast is spread upon sterilised solidified gelatine, prepared with or without nutritive solution (or on filter paper), and kept in a damp place; likewise in yeast-water and in sterilised water. Spore-forming cells may also occur in the films of the *Saccharomyces*.

characterised either by their mode of germination or by the form of their spores (*S. anomalous*, etc.).

After a lapse of time, dependent on the species, roundish particles of protoplasm appear in the cells; these are the first indications of spores (Fig. 52). In their further development they are surrounded by a wall, which is more or less clearly defined in the different species.

In most species the spores are spherical. *S. anomalous* forms an exception with its hemispherical spores, *S. Marxianus* and *S. fragilis* with kidney-shaped spores.

Two distinct types of germination may be distinguished. In the first type, to which *S. cerevisiae* I. belongs, the spores may expand during the first stages of germination to such an extent that the pressure which they exert on each other, while

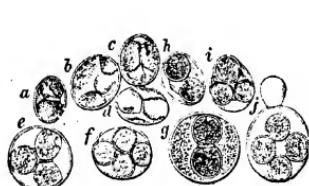


Fig. 52.—The first stages of development of the spores of *Saccharomyces cerevisiae* I. (after Hansen).—*a*, *b*, *c*, *d*, *e*, rudiments of spores, where the walls are not yet distinct; *f*, *g*, *h*, *i*, *j*, completely-developed spores with distinct walls.

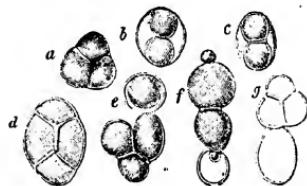


Fig. 53.—Spores of *Saccharomyces cerevisiae* I. in the first stages of germination (after Hansen).—At *a*, *d*, *e*, and *g*, formation of partition walls; *e*, *f*, and *g*, the walls of the mother-cells have become ruptured; *g*, a compound spore divided into several chambers, the coherent wall is ruptured in three places.

they are still enclosed in the mother-cell, brings about the formation of partition walls (Fig. 53). This is caused by the wedging or squeezing together of the protoplasm between the spores, or else the walls of the spores may be brought into close contact. During further development, a complete union of the walls may take place, so that a true partition wall results; the cell then becomes a compound spore divided into several chambers.

During germination (Fig. 54) the spores swell and the wall of the mother-cell, which was originally fairly thick and elastic, stretches out and consequently grows thinner. It is finally ruptured, and then remains as a loose or shrivelled

skin, partially covering the spores; or it may gradually be absorbed during germination.

Budding can occur at any point on the surface of the swollen spores; it usually takes place after the wall of the mother-cell has been ruptured or absorbed, but it also occasionally takes place within the mother-cell. After the buds have formed, the spores may remain connected, or they may soon break away from each other.

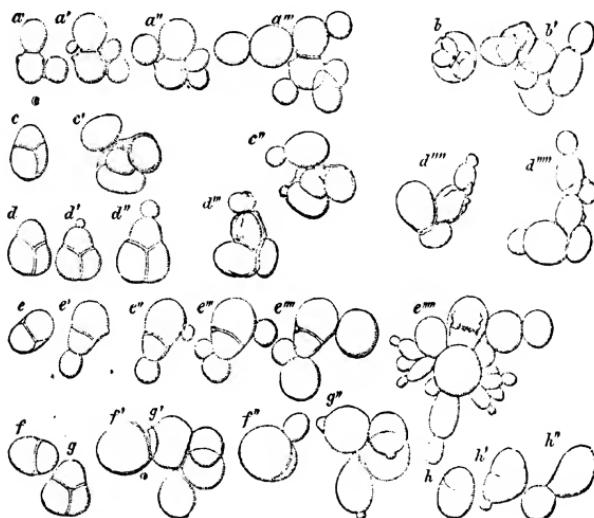


Fig. 54.—Budding of the spores in *Saccharomyces cerevisiae* L. (after Hansen).—a, Three spores without the wall of mother-cell; b, cell with four spores; c', the wall of mother-cell is ruptured; c, cell with four spores, three of which are visible; c' and c'' show the ruptured wall of mother-cell; d, cell with three spores; d''', the ruptured wall of mother-cell; e-e''', development of a very strong colony; f-h, other forms of development; h'', the wall between the two spores has disappeared.

Certain spores display a very remarkable behaviour (see Fig. 54, e-e'''' and h-h''); the absorption of the wall separating two neighbouring spores causing them to fuse together. It is possible that the biological significance of this phenomenon lies in the fact that the spores may thus have a greater chance of forming buds under unfavourable conditions. One spore plays the part of a parasite to the other. The amalgamation of the spore is, perhaps, the beginning of the process.

A similar fusion of spores was observed by Hansen in the

case of a wine yeast (*Johannisberg II.*). He placed spore-forming cells in a shallow layer of wort. In the course of a few hours they swelled up and burst the mother-cells. They were then transferred to a shallow layer in a saturated aqueous solution of calcium sulphate at 25° C. Under these conditions no budding took place, but several spores fused together and formed new endospores. Similarly, Guilliermond detected a fusion of spores in *S. Ludwigii*.

In this connection we may mention the fusion (copulation) of vegetative cells observed by Schiönning, Guilliermond, and Barker, in the case of *S. octosporus*, *Pombe*, *mellacei*, and *Zygosaccharomyces*. Further details are given in the systematic description of these species.

The germination of the spores of the known species of the groups *S. Pastorianus* and *S. ellipsoideus* takes place in essentially the same way as that just described.

*S. Ludwigii* forms a second and very different type (Figs. 55, 56), where germination does not take place through budding, but through a germinal tube, called a promycelium. Two such germinal tubes frequently fuse together, and the propagation of yeast cells takes place through division and not through budding, after the formation of a clearly defined septum. Similarly these yeast cells produce new cells. In this case, unlike the first type, it is not the spores, but the new formations springing from them that fuse together. Guilliermond observed such a fusion of germinal tubes in spores which were derived from different mother cells.

In older spores this curious fusion is more uncommon (Fig. 56). A few germ-filaments develop into a branched mycelium (group *b*).

The spores of *S. anomalus* have a remarkable shape similar to those of *Endomyces decipiens*.\* They are almost hemispherical with a rim round the base.

During germination the spores swell and the projecting rims may either remain or disappear. Buds then crop out at different points on the surface of the spore.

\* A fungus which is parasitic on the lamellæ of certain mushrooms. A similar species was described by P. Lindner as *E. fibuliger*.

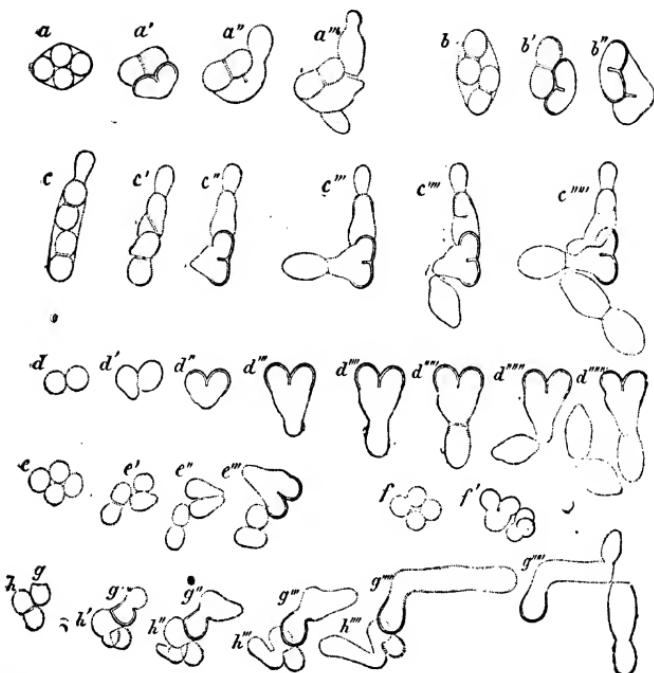


Fig. 55.—Germination of the spores of *Saccharomyces Ludwigi* (after Hansen).—a-c, Gypsum-block cultures twelve days old; d-h, a similar culture six weeks old.

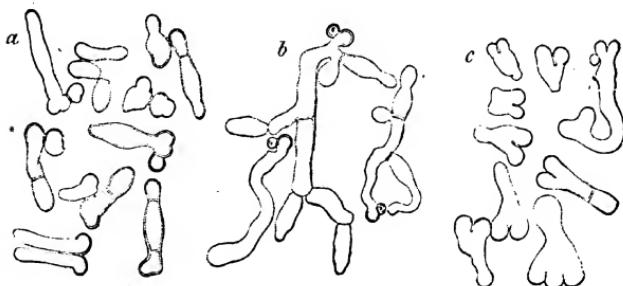


Fig. 56.—*Saccharomyces Ludwigi* (after Hansen).—Germinating spores from old gypsum-block cultures; a and b, each spore has developed a germ-filament; c, shows different forms produced by fusion.

One object of Hansen's work was to determine to what extent the formation of spores was influenced by different temperatures, with a view of ascertaining whether the various species behave alike, or whether it might not be possible in this way to discover characteristics. It was necessary to determine :—First, the limits of temperature—i.e., the highest and lowest temperatures at which spores can be formed; secondly, the optimum temperature—i.e., the temperature at which spores appear most rapidly; and, thirdly, the relation between the intermediate temperatures.

To determine the time required, the moment was registered at which the cells showed distinct indications of spores (compare Figs. 52 and 58). It is not possible to make use of ripe spores in these determinations, since no criterion exists for complete ripeness.

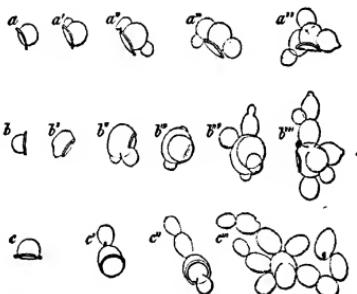


Fig. 57.—Germination of spores of *Saccharomyces anomalous* (after Hansen).

The results obtained by Hansen are as follows :—

The formation of spores takes place slowly at low temperatures, more rapidly as the temperature rises, until a point is passed at which their development is retarded and finally ceases.

The lowest limit of temperature for the six species first investigated was found to be  $0.5^{\circ}$ - $3^{\circ}$  C., and the highest limit  $37.5^{\circ}$  C. Hansen also determined the intermediate temperature and time relations for the six species, and found that when these two values are graphically represented, with the degrees of temperature as abscissæ, and the time intervals as ordinates, the curves are almost identical for the six species.

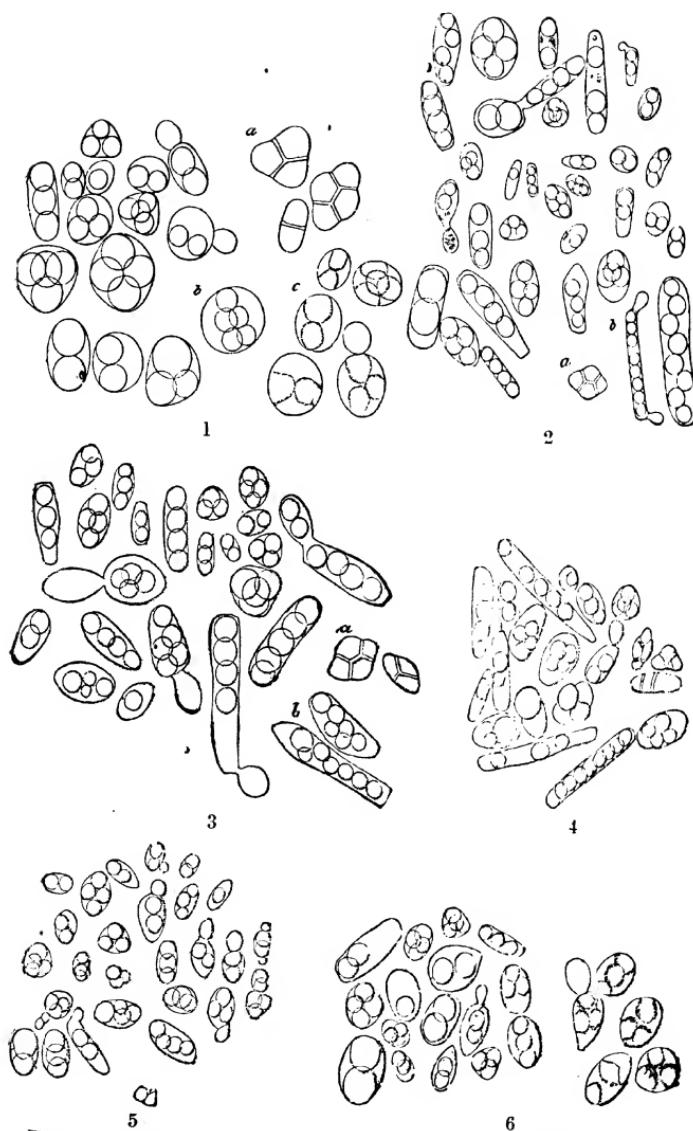


Fig. 58.—*Saccharomyces* with ascospores (after Hansen).—1, *Sacch. cerevisiae* I.; 2, *Sacch. ellipsoideus* I.; 3, *Sacch. Past.* II.; 4, *Sacch. Past.* III.; 5, *Sacch. ellipsoideus* I.; 6, *Sacch. ellipsoideus* II.; a, cells with partition-wall formation; b, cells containing a larger number of spores than usual; c, cells showing distinct rudiments of spores.

They sink from the ordinates of the lowest temperature towards the axes of the abscissæ, and then rise. At the same time, however, these curves indicate that the cardinal points determined, more especially for the highest and lowest temperatures, give characteristic distinctions for the different species—i.e., that the limits of temperature within which the formation of spores can take place differ for the various species (see systematic description).

In a course of years a number of investigators have carried out similar researches, including Holm, Will, Aderhold, Kayser, Seyffert, Marx, Schiönnung, and the author.

The following observations were made regarding the time required for the appearance of the first indications of spores in the six species maintained at the same temperature. At the highest temperature thirty hours were required for the development of each species; at 25° there was again no great difference in the time required; at the lower temperature, however, marked differences occurred. Thus, in the case of *S. cerevisiae* I., the first indications of spore-formation appear at 11.5° C. after ten days, but in the case of *S. Pastorianus* II., they appear within seventy-seven hours.

In all such determinations a considerable influence is exerted by the state of the cells, and the results vary with the temperature at which they have been grown, with their age, vigour, etc. (compare section on Variation of yeast cells). It follows that the composition of the nutrient fluid also exercises an influence. Thus in methodical, comparative experiments of this nature, it is a necessary condition that the previous cultivation of the cells should always be carried out in the same manner. If these external conditions are varied, the limits for the reactions of the species must be determined in each case.

By these experiments Hansen has established an important character for the determination of the Saccharomycetes. It is also of great interest to note that the spore-formation has a lower temperature maximum than budding, but a higher temperature minimum; in other words, spore-formation takes place within a narrower range of temperature than budding.

The method given below for the practical analysis of low brewery yeast was based by Hansen on the temperature curves

for the development of spores. Thus, it was found that at certain temperatures the species employed in the brewery, the culture yeasts, develop their spores later than the wild yeasts, several species of which occur as disease germs in the brewery. It is also important to note that the structure of the spores in these two groups is usually different. The young spore of culture yeast has a distinct wall or membrane; the contents are not homogeneous, but are granular, and exhibit vacuoles. In the case of wild yeast, on the other hand, the wall of the young spore is usually indistinct, the contents are homogeneous and strongly refractive. It should also be added that the spores of culture yeasts are usually larger than those of wild yeasts.

1. For the continuous daily control of low brewery yeast, as regards contamination with wild yeast, the following very convenient method is made use of:—At the conclusion of the primary fermentation, a small sample of the liquid is transferred from the fermenting vessel to a sterilised flask; this is set aside for some hours until the yeast has settled to the bottom, when the sediment is transferred to a gypsum block. It is then placed in a thermostat at a temperature of either 25° C. or 15° C.

It was shown that the species of culture yeasts employed in low-fermentation breweries can be divided into two groups. This has subsequently been confirmed by the elaborate investigations of Holm and Poulsen. At 25° C., one group yields spores at a later period than wild yeast, the other group produces spores in about the same time as wild yeast, but at a temperature of 15° C. the cells of wild yeast show spore-formation much sooner than the cells of either group of culture yeasts.

The cultures maintained at 25° C. are examined after an interval of forty hours, and those maintained at 15° C. after an interval of three days.

The author has shown that high brewery yeasts can be analysed in a similar manner. In the case of some species, however, the analysis is best made at 10°-12° C., because a well-marked difference of time between the beginning of spore-formation in culture yeast on the one hand and wild yeast on the other can only be observed at this temperature.

According to the author's researches, distillers' yeast may be analysed in the same way. Lower temperatures are to be preferred for this analysis. Often, however, the investigation into the construction of the spore in the selected yeast-type must form the chief part of the analysis, the difference of time for spore-formation in culture yeast and wild yeast frequently proving inadequate.

Aderhold has established the fact that wine yeast, like beer yeast, may be analysed by Hansen's method.

By means of experiments undertaken to determine to what extent Hansen's analytical method can be relied on for technical purposes in low-fermentation breweries, Holm and Poulsen concluded that a very small admixture of wild yeast, about  $\frac{1}{200}$ th of the entire mass (Carlsberg bottom-yeast No. 1), can be detected with certainty. Hansen's previous researches had shown that when the two species, *Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*—which are capable of producing yeast-turbidity in beer—are present to the extent of only 1 part in 41 of the pitching yeast, the disease is not developed, provided that the normal conditions of fermentation and storage have been maintained. Further, *Sacch. Pastorianus I.*, which imparts to beer a disagreeable odour and an unpleasant bitter taste, can scarcely exert its injurious influence under the same conditions when the admixture of this yeast amounts to less than 1 part in 22 of the pitching yeast. Consequently, Hansen's method for the analysis of yeast by means of ascospore formation is sufficient to establish its purity.\*

When the object of the analysis is to characterise the different species present in the sample with greater accuracy, a number of cells are isolated by fractionation, and each of the growths obtained is separately examined.

In an investigation of bottom yeast in the different stages

\* It is obvious that such an analysis from the vat does not enable direct conclusions to be drawn regarding the predominant biological conditions existing during secondary fermentation in the cask. If, for instance, the beer is run off with a very small quantity of yeast, even if the infection is a small one, the wild yeast will chiefly be found floating in the liquid, and will be carried over into the cask, whilst the greater part of the culture yeasts will have sunk to the bottom of the vat.

of the primary fermentation, published by Hansen in 1883, it was shown that the young cells of wild yeasts are present in largest amount during the last stages of primary fermentation and in the upper layers of the liquid. The samples taken from the fermenting vessel for the analysis of yeast must, therefore, be taken during the last few days of the primary fermentation. If a dried or partially dried sample of yeast is to be examined, it must first be transferred to wort, and one or more fermentations must be completely carried out with it.

The rule that wild yeasts develop only in the more advanced stages of fermentation applies also to top-fermentation yeast as used in breweries. This was shown by numerous analyses of beer from Danish, English, French, and German breweries carried out in the author's laboratory. As is well known, it was this very appearance of wild yeast in English top-fermentation breweries which gave rise to the erroneous view that such species are necessary for conducting a normal secondary fermentation.

2. The analysis of the yeast in the propagating apparatus, which must be absolutely pure, is carried out as follows:—At the conclusion of fermentation, samples are withdrawn, with every precaution, into Pasteur flasks or into the Hansen flasks employed for sending out yeast samples; from these, small quantities are introduced into flasks containing neutral or slightly alkaline yeast water or yeast-water dextrose, and maintained at a temperature of 25° C., the object being to test the yeast for bacteria. The remainder is set aside to allow the yeast to settle, the beer is decanted, and an average sample of the sediment is introduced into a cane-sugar solution containing 1 to 4 per cent. of tartaric acid. After three or four cultivations in such a solution it is further cultivated a few times in beer-wort, and then tested for spore-formation. The smallest traces of wild yeast in the apparatus are brought into a state of vigorous development by this treatment.\*

\* It is evident that this method is not available for the analysis of ordinary yeast, because the cultivation in the tartaric solution will cause the wild yeast cells to increase very considerably in number, and consequently render it impossible for the analyst to judge of the degree of contamination.

## I. SACCHAROMYCES.

The name *Saccharomyces* is used to distinguish budding fungi with endogenous spore-formation. The great majority of species are only known in this form, but a few can develop a mycelium. In the case of one particular group of *Schizosaccharomyces* division of the cell takes place instead of budding, exactly in the same way as with certain of the mould fungi.

In addition to these fungi many other kinds of budding species occur in nature which do not display endogenous spore-formation. Thanks to investigations by de Bary, Zopf, Brefeld, and others, it is now known that certain of these are developed from the higher fungi *Ustilagineæ* (smut-fungi), *Basidiomycetes*, etc.

A glance at the following figures shows that the *Saccharomyces* may develop mycelial cells in their films. Thus cultures of *S. Marxianus* may occur with typical branched mycelium. Such formations may probably be regarded as tending to show that if these fungi are afforded more favourable conditions of development in nature than those obtaining when they are artificially cultivated in a laboratory, they are likely to develop as typical moulds. The following observations of the author appears to favour this view:—On dried grapes, growths of *Dematioides*-like moulds have been observed with a rich formation of spores (see Fig. 45).\* If such growths are cultivated either in or upon a substratum in flasks, their spores develop only budding cells with endogenous spore-formation. In the same way vigorous growths of mould have been found on slices of Agave stems from Mexico, which at first suggest *Monilia*, and give a strong formation of spores. By cultivation in nutritive liquids and on gelatine only *Saccharomyces* cells are produced. On saccharine material received from Jamaica, growths of moulds were found resembling *Oidium*, but the cells also exhibit spore-formation, and by further development

\* The fungi do not possess the characteristic coloured and thick-walled resting cells of *Dematioides pullulans*.

of the mould in and upon sterile substrata nothing but a growth of *Schizosaccharomyces* is obtained, and no mycelium.\*

In all three cases such substrata were utilised as had otherwise proved favourable for the growth of moulds. But it was impossible, under laboratory conditions, to reproduce the natural conditions which favour the formation of these *Dematiuim*, *Monilia*, and *Oidium*-like fungi.

Further investigation will determine how far such conditions are to be found in nature. These observations, at any rate, show that there are cases where the natural conditions allow of a development which cannot be substantiated by artificial conditions in the laboratory, and the conclusion appears to be warranted that other fungi, including higher fungi, may behave in the same way, like the *Ustilagineæ* and other forms which regularly reproduce budding growths, which are incapable of forming endospores. An excellent but isolated example of the development of *Saccharomyces* cells from a fungus, *Glæosporium*, belonging to a higher system, has been recorded by Vialla and Pacottet.†

The basis of a scientific system of classification was suggested by Hansen in 1904 as follows :—

#### Family—*Saccharomycetes*.

Budding fungi with endospores and vigorous formation of yeast cells. Typical mycelium only occurs in few cases. Every cell may occur as the mother-cell of a spore. Spores unicellular. Number of spores in each mother-cell usually from one to four, seldom up to twelve.

\* As stated in the section on Variation, Lepeschkin observed a similar weak formation of mycelium in individual cells of *S. mellacei*.

† Whatever may be the much-discussed genetic connection between *Aspergillus Oryzae* and *Saccharomyces*, it is certain that the conidia of many individuals have been observed to bud. How far a mistake may have occurred in transferring these budding growths to gypsum blocks, where they showed endogenous spore-formation, it is no longer possible to say. All subsequent experiments with growths of *Aspergillus* species have given negative results.

## A. TRUE SACCHAROMYCETES.

## 1st Group.

The cells immediately form sedimentary yeast in saccharine nutritive liquids, and only at a much later stage form a film with slimy growth and without inclusion of air. Spores smooth round, or oval, with one or two membranes. Germination by budding or by the formation of germinal tubes (promycelium). All, or at any rate the greater number, of this group bring about alcoholic fermentation.

GENUS I.—*Saccharomyces* Meyen.

The spores provided with one membrane germinate by budding. In addition to formation of the yeast cells a few give mycelium with distinct transverse walls.

(To this genus belong the culture yeasts and the great majority of wild yeasts.)

GENUS II.—*Zygosaccharomyces* Barker.

Distinguished by the copulation of cells. In other respects identical with the preceding genus.

GENUS III.—*Saccharomyces* E. C. Hansen.

By germination of the spores, possessing one membrane, form a promycelium. From these, as well as from the vegetative cells, budding takes place with incomplete separation. Formation of mycelium with distinct transverse walls.

GENUS IV.—*Saccharomyces* Schiönnung.

The spores possess two membranes, otherwise the characters, so far as they are known, are identical with those of *Saccharomyces*.

## 2nd Group.

The cells immediately form a film in saccharine nutritive liquid, which is dry and opaque on account of the inclusion of air, and can readily be distinguished from the film-formation of the first group. The spores are hemispherical, angular,

hat-shaped or lemon-shaped ; in the last two cases provided with a distinct projecting rim ; otherwise smooth. They have only one membrane ; germination takes place by budding. The majority of species are distinguished by the formation of esters ; a few do not bring about fermentation.

GENUS V.—*Pichia* E. C. Hansen.

The spores hemispherical or irregular and angular. No fermentation ; strong growth of mycelia.

GENUS VI.—*Willia* E. C. Hansen.

Spores hat-shaped or lemon-shaped with distinctly projecting rims. The majority produce esters vigorously ; a few produce no fermentation.

B. DOUBTFUL SACCHAROMYCETES.

*Monospora. Nematospora.*

The genus *Schizosaccharomyces* cannot be included in the family of Saccharomycetes.

1. THE SPECIES USED INDUSTRIALLY (CULTURE YEASTS).

(a) **Brewery Yeasts.**

According to the physical phenomena of fermentation, a distinction is made between low- and high-fermentation yeasts, both in the brewery and elsewhere. The low-fermentation yeasts gradually collect during fermentation to form a deposit in the fermenting liquid, whereas the top-fermentation yeasts, in the normal course of fermentation, partly form a layer on the surface of the liquid, differing in character and thickness according to the race, and partly form a deposit. The two kinds of yeasts in the brewery impart a different character to the fermented liquor. This has been established by parallel experiments with wort of identical composition. The two groups of yeast may, therefore, be said to exhibit a different form of chemical activity. Bau has proved that most of the known species of low-fermentation yeasts ferment melibiose, whilst some of the top yeasts are incapable of fermenting it.

According to Bau and Fischer, melibiase, the enzyme that ferments melibiose, could only be detected in low-fermentation yeasts, and not in those top yeasts that are unable to ferment melibiose.

According to Hansen's recent work (referred to in the section on Variation), individuals may occur in old cultures of bottom yeasts which exhibit top-fermentation phenomena, and similarly, if in smaller numbers, individuals in top yeasts which behave like bottom yeasts.

After Hansen had introduced a pure culture of bottom yeast into the Carlsberg brewery in Copenhagen it was possible to discover how extensive and how deep-seated the differences are which distinguish the various bottom-fermentation brewery yeasts. With this object in view the writer undertook a long series of comparative experiments with pure cultures of top and bottom yeasts from every part of the world, noting in particular the degree of fermentation, the clarifying power of the liquid, the physical phenomena of fermentation, and the stability of the fermented liquor. As early as 1886, in the first edition of this book, he set forth a classification of typical species or races, the correctness of which has been confirmed by subsequent workers in this field.

#### A. BOTTOM-FERMENTATION SPECIES.

1. Species which clarify very quickly and give a feeble fermentation ; the beer holds a strong head. The beer, if kept long, is liable to yeast-turbidity. Such yeasts are only suitable for draught beer.
2. Species which clarify fairly quickly and do not give a vigorous fermentation ; the beer holds a strong head ; high foam : the yeast settles to a firm layer in the fermenting vessel. The beer is not particularly stable as regards yeast-turbidity. These yeasts are suitable for draught beer and some for lager beer.
3. Species which clarify slowly and attenuate more strongly. The beer is very stable to yeast-turbidity. These yeasts are suitable for lager beer, and especially for export beers.

## B. TOP-FERMENTATION SPECIES.

1. Species which attenuate slightly and clarify quickly.  
The beer has a sweet taste.
2. Species which attenuate strongly and clarify quickly.  
Taste of beer more pronounced.
3. Species which attenuate strongly and often clarify slowly.  
The beer is stable to yeast-turbidity.

By far the greater number of high-fermentation yeasts examined in this respect are able to carry through a secondary fermentation. In class 2, and especially 3, the secondary fermentation is very vigorous and long continued.

Before the results of these comparative experiments had been published, both Hansen and the author had had the opportunity, as will be seen, of demonstrating that many of the species so characterised appeared as strongly marked types when applied in the form of mass cultures in practice, and that both in the above and other respects typical differences made their appearance between the individual races or species which found application as culture yeasts. The experience gained during the years that have since elapsed goes to prove that by a methodical selection of a race, an element of certainty is introduced into the fermenting conditions, which was impossible when a mixed yeast of unknown composition was employed.

In 1884 Hansen made the following pronouncement :—

“ We find by closer investigation that differences exist amongst the kinds of yeast which must be described as good from the standpoint of the brewer. Thus, under similar conditions, some give a quicker and more complete clarification in the primary fermentation and a more feeble attenuation than others. Again, differences are found in respect to flavour. If my method is followed, it is possible, nevertheless, to select with care and quite methodically, that species which is best suited for the particular work. This phase of the question has been practically solved at Old Carlsberg, where a yeast has been selected, in addition to that previously described, which is better suited for making lager beer, whilst the former is better for export beer. Where the fermentation industry

formerly groped blindfold, and everything was a matter of guess work, a path has now been opened to a technique."

Hansen is here referring to the two races of yeast that were first isolated and described.

To what extent individual types display a pronounced character in their practical application depends largely on the nature of the treatment. Thus the degree of fermentation is determined both by the composition of the liquid and by the other conditions of fermentation. A race which gives a vigorous fermentation, can obviously only display this property under certain conditions. On the other hand, typical characters exist which may become noticeable under very different external conditions. Thus it was shown by the author, in the earliest stages of the development of this important reform, that top-fermentation species from the brewery, which have a definite influence on the odour and flavour of the fermented liquor, can be recognised by this fact when they are used in breweries in distant countries, where both the raw materials and the methods of working may be entirely different. The same applies when such species are introduced in absolutely pure cultures, which, beginning on the small scale with one or two litres of thin yeast liquor, are propagated by degrees in brewery wort, and thus adapted to it.

The two first races obtained as pure cultures, referred to by Hansen in the above quotation, were—*Carlsberg No. 1*, a yeast applied for many years in the Carlsberg brewery in Copenhagen, and *Carlsberg No. 2*, which was introduced from a German into a Copenhagen brewery, the fermentation being under the control of the author. After he had drawn attention to the remarkable fermentation phenomena observed with this yeast, which differ widely from that of *Carlsberg No. 1*, it was introduced into the Carlsberg brewery and isolated as a pure culture by Hansen and the author.

In 1885 the author had the opportunity of answering the question whether different races or species of *S. cerevisiae* exist, the answer being based upon his own investigations of these two species.

The first race chiefly exhibits slightly elongated cells, amongst which somewhat smaller pointed individuals distinguished by granular contents are not infrequently found. If the yeast is taken from the fermenting vessel, washed with water, and placed for a short time on ice, it will be observed that the whole cell content rapidly changes to a granular structure, and if maintained under these conditions for several days, it will be found that the number of dead cells rapidly increases. The second race behaves in quite a different way. The cells are short and oval, or almost spherical, under normal conditions in the fermenting vessel; only a few bent individuals are observed, and in a washed condition the cells retain their clear or slightly granular contents for a long time; very few dead cells are observed even after long preservation in this condition.

If each of the growths is placed on moist gypsum blocks, maintained at the same temperature, and their further development observed from day to day, it will be seen that the two races behave quite differently, assuming that the temperature lies within the limits for the growth of spores. *Race II.* forms fully ripe spores at a time when *Race I.* does not show a trace of these organs of propagation.

The following distinctions are of value in practice in determining the two races:—

In order to obtain the normal course of the primary fermentation it is essential that *Race I.* should be introduced at a somewhat higher temperature ( $7.5^{\circ}$  C.) than *Race II.* Larger quantities of *I.* than of *II.* must be used for pitching, in the proportion of 66 to 58. The time of setting and of frothing naturally differs. Both phenomena appear to occur somewhat earlier with *Race II.* than with *Race I.*

The nature of the frothing and the coating of yeast differ greatly. *II.* gives a strong high head and a dense coherent cover; *I.* a low head, and the liquid often shows bald patches. Moreover, *Race I.* gives a very lasting fermentation, and, as a consequence, a slower clarification than *Race II.*, which when pure gives a particularly bright clarification. The sedimentary yeast in the vat lies more compactly, and the colour of *II.* is somewhat lighter than that of *I.*

The attenuation during the primary and secondary fermentation with normal wort and in the same brewery is stronger with *Race I.* than *II.*

With regard to the finished beer, similar differences are noted, particularly regarding flavour and resistance to turbidity. Most experts prefer *II.* for flavour, but some difference of opinion exists. It is otherwise regarding the stability of the beer, especially with regard to yeast turbidity. In this respect the difference is very marked. *I.* gives a quite exceptionally stable beer, and is specially suitable for export beer, which when fermented with this yeast remains unaltered for about a month without any further treatment, and by mild pasteurisation is rendered stable for much longer periods. *Race II.*, although it displays much finer phenomena during the primary fermentation, is unable to produce completely stable beer (about ten days in bottles at room temperature), and it is also noteworthy that this race is much less resistant to wild yeast than *I.* On account of the rapid clarification and quick fermentation of the liquor this race is adapted for beers which are to be stored for a short time, and are to be consumed immediately.

In general, it may be stated that the whole of the differences indicated have been observed for years in different breweries, and that they are so sharply defined that every brewer could at once distinguish the two yeasts with certainty when they have been put into the fermenting vessel, and could foretell the nature of primary and secondary fermentation. In fact, no one could be in doubt that we are dealing with two truly distinct races or species.

In the detailed descriptions of these two races (see Figs. 59 and 60), published by Hansen in 1888 (which might equally well have appeared before the author's publications, as will be seen from the preceding historical description), the characteristic distinctions between these two species are further emphasised. Amongst other observations, reference is made to the giant cells, remarkable and abnormal large round cells which suggest the cells of *Mucor* yeasts.

In 1908 Hansen described further typical differences, and gave the species the names *S. Carlsbergensis* and *S. Monacensis*.

*S. Carlsbergensis* (= No. 1) has temperature limits for budding in wort at about 33.5° C. and 0° C. At the maximum temperature the cells are considerably larger than at the outset, but have approximately the same shape; giant cells are numerous. At the minimum and up to 9° C. many of the cells assume the sausage-shape, and form large mycelial colonies. In the films elliptical and round cells principally occur. The giant colonies are rosette-shaped with a depression, and less frequently a distinct knot in the middle, with concentric rings and radial streamers; they have a smooth or scaly surface and wavy outline. The colonies in the usual plate-cultures

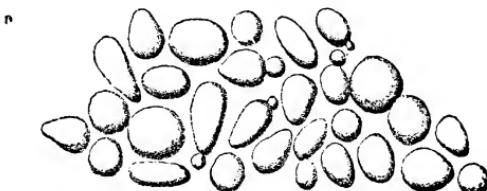


Fig. 50.—Carlsberg low-fermentation yeast No. 1 (after Hansen).

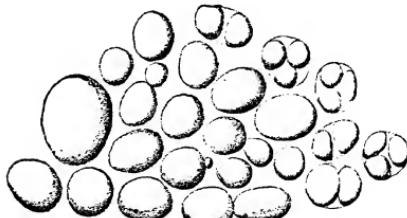


Fig. 60.—Carlsberg low-fermentation yeast No. 2—a few cells with spores (after Hansen).

are built up as small pin heads with a light greyish-yellow and waxy appearance.

*S. Monacensis* (= No. 2) has temperature limits for budding at about 33° and 1°. At the maximum the cells are larger, and especially longer, than at the outset, and at the minimum and up to 9° C., in contrast to the former species, it develops colonies consisting principally of spherical and elliptical cells. In dextrose-yeast-water, the giant cells may assume huge dimensions. In the film, the cells are spherical and elliptical. The giant colonies and the small colonies agree in appearance with those of the former species.

As an admirable example of the application of Hansen's biological methods to the differentiation of yeast species, we give the comparison of four low-fermentation brewery yeasts carried out by Will. This, again, emphasises the fact that, within this group, just as clearly distinguished species occur as in those groups of *Saccharomycetes* which have not yet found an industrial application. Will began his characterisation by adopting the classification of brewery-yeast types published by the author in 1886. He classed *Race 93* and 2 as high-fermenting, *Race 7* as a low-fermenting type, and *Race 6* as a yeast of intermediate fermentation.

The four yeasts can be distinguished as follows:—

*Race 2* has large roundish or oval cells; the colonies on gelatine are spherical or lenticular; the spores are formed easily and freely; spore-formation takes place between 31° and 11°; the optimum is 25°-26°; film-formation occurs between 31° and 7°; very slow.

*Race 6*; oval cells predominate, but the species has a great tendency to form sausage-shaped cells; colonies on gelatine are spherical or lenticular; the spores form easily and freely; spore-formation occurs between 31° and 11° C.; the optimum is 28°; film-formation between 31° and 7°, occurring later than with *Race 7*.

The cells of *Race 93* are typically oval with a tendency to assume a roundish shape. The colonies in gelatine are spherical or lenticular; spores are freely and easily formed; spore-formation occurs between 30° and 10° C.; optimum 28°; film-formation between 31° and 4°; very feeble and slow; resting cells occur freely in the film.

*Race 7* has oval cells which closely approach the spherical shape; giant cells regularly occur, and at the end of the fermentation large budding colonies with small oval cells frequently occur; the young colonies on gelatine are irregular with a marked wavy and fringed outline; the species develops spores with great difficulty; spore-formation occurs between 30° and 13°; optimum 25° to 26°; film-formation between 28° and 4°; appears earlier than with the other species: resting cells are to be found only in small numbers in the film.

P. Lindner distinguished two species of low-fermentation yeast in 1889, which he called "Saaz" (weakly fermenting) and "Frohberg" (strongly fermenting). These names have been adopted in the literature as a description of weakly and strongly fermenting yeast types in general. The investigation of these groups was subsequently undertaken by Delbrück, Reinke, Irmisch, and others.

A thorough description of two low-fermentation yeasts of the Frohberg type (D and K) has been given by Schönfeld and Rommel. D gives longish, almost sausage-shaped cells, K predominantly spherical and oval cells. D is more inclined to form spores than K. In hanging drops, differences in the shape and size of the cells can be remarked. K forms budding colonies more rapidly and in greater number. In the growth of giant colonies similar differences have been observed. The content of albumen, the percentage of ash and phosphoric acid are higher in K than in D, and K has a higher specific gravity than D. Auto-digestion occurs more rapidly with K. K has a higher "raising power," and is more sensitive to high temperatures. Fermentation sets in more rapidly with K. At temperatures above 30° the fermentative activity of K yeast is weakened to a much greater extent than that of D. The film growth of K is capable of fermenting more carbohydrate than the sedimentary yeast, whilst with D the difference is unimportant. In the brewery, K ferments 10 per cent. higher than D in the fermenting vessel, and gives a lighter coloured beer. The final fermentation is identical with the two species.

In 1883-85 very detailed researches were carried out by the author in elaborating the principle laid down by Hansen, and introduced in the Carlsberg brewery, the application of methodically selected pure cultures derived from a single cell. The experiments were carried out with a view of securing practical conditions, and the results gained in the laboratory were applied on the large scale in breweries in many European countries. The reform found acceptance by prominent fermentation technologists (in the early stages, especially by Thausing, Lintner, and Aubry), and it was gradually incorporated into the courses of all zymotechnological institutes.

***Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* I.  
Hansen.**

This species, described in 1883, is an old English top-fermentation yeast which is in use in London and Edinburgh breweries.

The young growth of sedimentary yeast developed in wort consists essentially of large round and oval cells; truly elongated cells do not occur under these conditions.

Ascospore-formation (Figs. 52 to 54, and 58, 1) :—\*

At  $37.5^{\circ}$  C. no ascospores are developed.

36-37 the first indications are seen after 29 hours.

35	„	„	25	„
33.5	„	„	23	„
30	„	„	20	„
25	„	„	23	„
23	„	„	27	„
17.5	„	„	50	„
16.5	„	„	65	„
11-12	„	„	10 days.	

9 no ascospores are developed.

Wall of spores very distinct. Size of spores  $2.5$  to  $6 \mu$ .

Film-formation :—

At  $38^{\circ}$  C. no film-formation occurs.

33-34 feebly-developed film specks  
are seen after . . . 9-18 days.

26-28	„	„	7-11	„
20-22	„	„	7-10	„
13-15	„	„	15-30	„
6-7	„	„	2-3 months.	Fig. 62.

5 no film-formation occurs.

Microscopical appearance of the cells in the films :—

At  $20^{\circ}$  to  $34^{\circ}$  C.; colonies frequent; sausage-shaped and curiously formed cells occur.

\* The preparatory treatment of a *Saccharomyces* species for these investigations must be made in the following manner:—After the cells have been cultivated for some time in ordinary wort ( $14^{\circ}$  Ball.) at the ordinary room temperature, the vigorous young cells obtained are introduced into fresh wort, in which they are allowed to develop for about twenty-four hours at  $25^{\circ}$ - $27^{\circ}$  C. This growth is used for the gypsum-block culture.

At 15° to 6° C. (Fig. 62).—The greater number of the cells resemble the original cells; isolated abnormal forms occur.

In old cultures of films all forms occur, including extremely elongated mycelial cells (Fig. 63).

The temperature limits for budding in wort are 40° C. and 1° to 3° C. The species develops invertase and maltase; it ferments saccharose, maltose, and dextrose, but not lactose. It produces a vigorous fermentation in beer-wort.

The first series of pure top-fermentation species were prepared by the author in 1884 from material collected in many

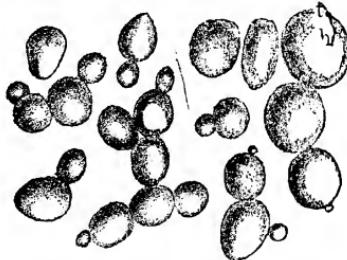


Fig. 61.—*Saccharomyces cerevisiae* I. (Hansen).—Cell-forms of young sedimentary yeast (after Hansen).



Fig. 62.—*Saccharomyces cerevisiae* I. (Hansen).—Film-forms at 15° to 6° C. (after Hansen).

European countries, with the object of introducing such pure, selected types, developed from single cells, into practice. It was soon seen that the typical differences between the species were much more pronounced than is the case with low-fermentation yeast. It was found that one group of the species used in breweries had an extraordinarily weak fermentative activity. The fermentation ceased, under the conditions existing in the breweries, when 1 to 2 per cent. of alcohol had been formed in beer-wort; the main mass of the yeast usually spread out over the surface of the liquid to form a coherent layer.

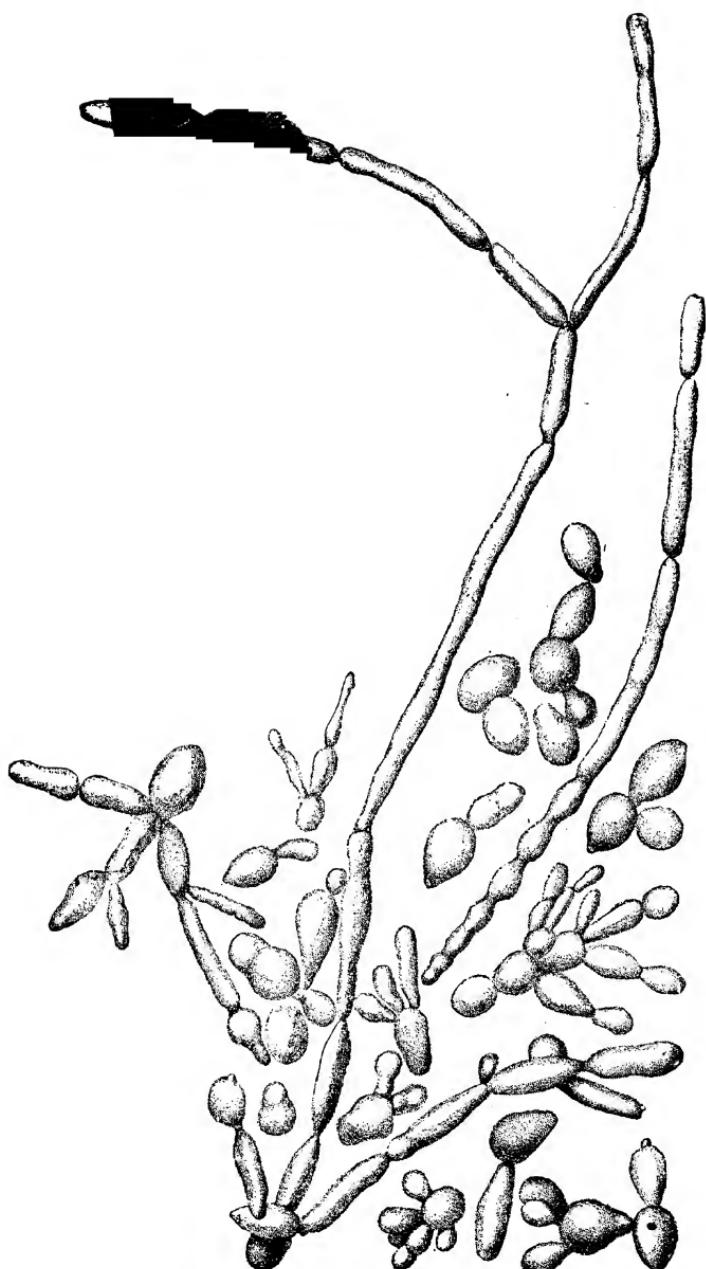


Fig. 63.—*Saccharomyces cerevisiae* I. (Hansen).—Cell-forms in old cultures of films  
(after Hansen).

Species of the second group behave quite differently. Under similar conditions fermentation can be carried on for a much longer time, clarification goes on slowly, and when the primary fermentation is at an end the beer is decanted from the yeast, which to a large extent is sedimentary yeast; a secondary fermentation takes place, the duration varying according to the species.

As representatives of the first group, the species chiefly used in Danish breweries may be mentioned, and of the second group, many of those applied in English breweries.

The purely-cultivated Danish, top-fermentation species fall into two distinct types, according to their chemical activity. The first impart a decidedly mild flavour; the fermentation is weak without noticeable secondary fermentation, and the layer of yeast forming on the surface of the liquid is loose and slimy. The second type gives a strongly pronounced flavour, the fermentation is stronger, with subsequent secondary fermentation, and the layer of yeast has a dense consistency. By long-continued use of both types the latter has proved to be more generally acceptable.

The English species that have been subjected to examination, and have been proved to bring about a distinct secondary fermentation, exhibit a great variety of form and various construction of spores. Many of these differences have been recorded, both in these respects and in relation to fermentation. According to the observations\* of J. C. Holm and the author, the following facts have been established:—

The formation of cells at the different stages of alcoholic fermentation was determined by growths which were first kept for a long time in a 10 per cent. sugar solution, then grown for several generations in beer-wort, and finally developed for twenty-four hours in Pasteur flasks at 25° C. The development of the films and their appearance to the naked eye were studied in growths in Erlenmeyer flasks at room temperature (about 20° C.). Growths in Pasteur flasks at room temperature were made use of for determining the physical character of the sedimentary yeast. The fermentation experiments were

\* Published for the first time in *Micro-Organisms and Fermentation*, 3rd edition, 1900.

carried out at room temperature in sterilised, hopped wort contained in tall cylindrical glasses covered with several layers of filter paper. After the primary fermentation was completed, the liquids were poured into sterile flasks and allowed to stand at low temperatures. The amount of alcohol was determined at the completion of the primary fermentation and again after the first fortnight of the secondary fermentation, and, lastly, after the following fortnight. The primary fermentation was interrupted when the appearance of the cells showed that the first vigorous development had ceased. In this comparison no attempt was made to decide what quantity of alcohol could be produced by the species during primary and secondary fermentation. The object was simply to institute a comparison.

The flavour of the fermented liquor was recorded after the beer had undergone a secondary fermentation at a low temperature in flasks closed at first with cotton-wool and afterwards with ground-glass stoppers.

1. (*Fig. 64, a, and Fig. 65, a.*)

The cells during fermentation are comparatively small, oval, and linked in chains; among them occur big, round and grotesque forms.

The yeast lies rather loose in the flask; if shaken it does not distribute itself equally in the wort, but separates into clots.

Film-formation: After a lapse of 31 to 32 days a very thin film covers almost the whole surface of the liquid.

The cells of the film are of about the same size as those seen during the primary fermentation; some cells much elongated.

The spores, if developed at a low temperature, are small, full of vacuoles, and slightly granulated; as a rule, only one or two in each cell.

At 11° to 12° C. a few spores make their appearance on the seventh day; at 25° C. abundant development of spores in forty hours.

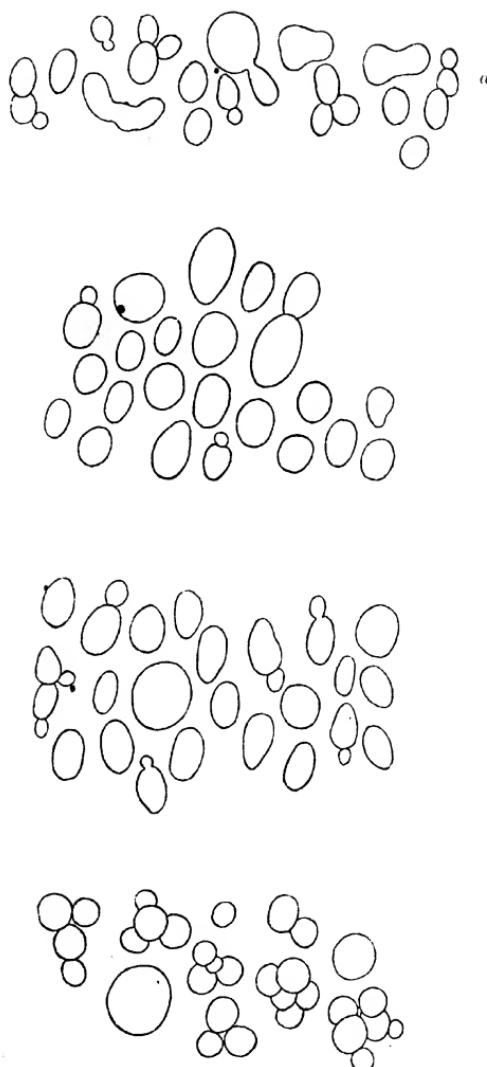


Fig. 64.—*a-d*, Young growths of English top-yeasts (Holm).

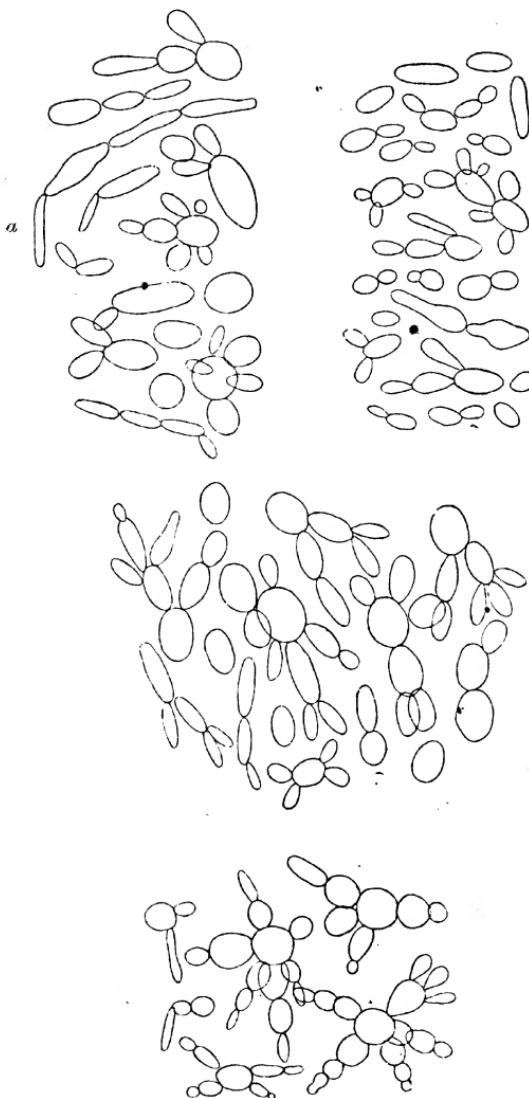


Fig. 65.—*a-d*, Film-formations of English top-yeasts (Jörgensen).

When the principal fermentation was broken off, the liquid contained 2.49 per cent. by vol. of alcohol ; during the two following periods (see above) 0.31 and 0.57 per cent. by vol. were produced.

Production of acid, after expulsion of  $\text{CO}_2$ , corresponds to 5 c.c. of decinormal caustic soda solution.

The fermented liquid has an agreeable smell and a fine aromatic taste.

2. (*Fig. 64, b, and Fig. 65, b.*)

During fermentation most cells are free, medium-sized, round and oval ; among them there occur round and oval giant cells.

The yeast lies loose in flask ; if shaken slightly, it is distributed like a cloud throughout the liquid.

**Film-formation :** After 31 to 32 days, a few large patches.

The cells of the film are smaller than those seen during primary fermentation ; ellipsoidal and slightly lengthened.

The spores are big, if developed at a low temperature ; formation of partition walls readily occurs.

At 11° to 12° C. very few spores occur on the seventh day ; at 25° C. rather abundant spore-formation in forty hours.

When the principal fermentation was broken off, the liquid contained 2.3 per cent. by vol. of alcohol ; in the two following periods 1 and 0.46 per cent. by vol. were formed.

**Acid-production :** 6.

Disagreeable smell and taste.

3. (*Fig. 64, c, and Fig. 65, c.*)

During fermentation the growth shows free cells and small chain-formations of oval forms ; a few globular giant cells.

The yeast lies very compact in flask ; it partially rises in the liquid only when violently shaken.

**Film-formation :** In 31 to 32 days the growth forms a very thin film, which does not cover the entire surface of the liquid.

Some of the cells of the film have the same size and shape as those seen during primary fermentation ; others are slightly lengthened.

If developed at a low temperature, the spores are of very varied size, with comparatively feeble refractivity, and without distinct vacuoles. Partition-wall formations occur. At 11° to 12° C., in seven days, only rudiments of spores appear; at 25° C., in forty hours, spores are very freely formed.

When the principal fermentation was broken off, the liquid contained 2.26 per cent. by vol. of alcohol; during the following two periods 0.79 and 0.00 per cent. by vol. were formed.

Acid-production : 5.5.

Disagreeable smell and taste.

4. (*Fig. 64, d, and Fig. 65, d.*)

During fermentation, colonies consisting of many small spherical cells occur, and among these spherical giant cells.

The yeast lies loose in flask; if slightly shaken, it is distributed like a cloud throughout the whole liquid.

Film-formation : After 31 to 32 days, only the merest trace.

The cells of the ring-growth occur in colonies, which sometimes contain upwards of a hundred cells, all derived from a single cell; the youngest growths are elongated and very narrow.

The spores, if developed at a low temperature, are small and vacuolised. At 11° to 12° C., even after a fortnight, no spore-formation; at 25° C., for forty hours, a very scanty development of spores.

When the principal fermentation was broken off, the liquid contained 1.8 per cent. by vol. of alcohol; during the following two periods 1 and 0.82 per cent. by vol. were formed.

Acid-production : 5.5.

Disagreeable smell and taste.

5. (*Fig. 66, a, and Fig. 67, a.*)

During fermentation most cells are free, medium-sized, and oval.

The yeast lies rather loose in flask; if shaken, it is not distributed equally in the wort, but separates into clots.

Film-formation : After 31 to 32 days a distinct film, which, however, does not cover the whole surface, and subsequently develops slowly.

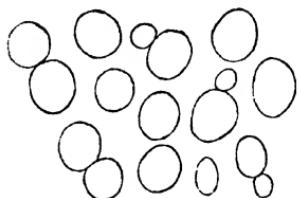
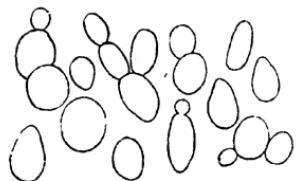
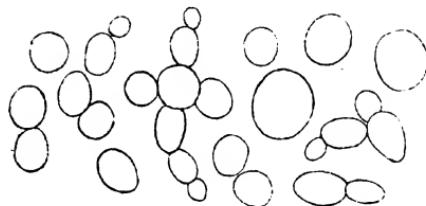
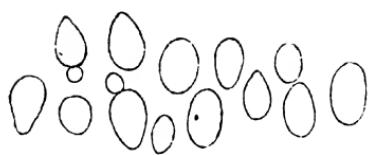


Fig. 66.—*a-e*, Young growths of English top-yeasts (Holm).

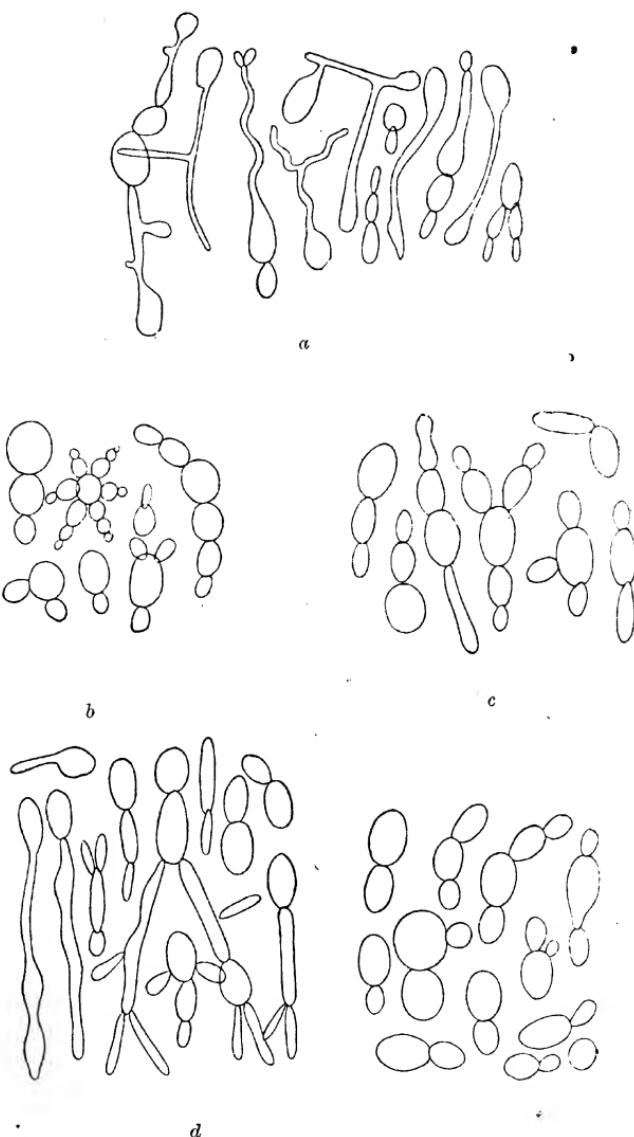


Fig. 67.—*a-e*, Film-formations of English top-yeasts (Jørgensen).

The cells of the film have a very different appearance from those seen in the fermentation-stage. Many of them are much lengthened and irregularly twined ; some have developed a ramified mycelium.

If developed at a low temperature, the spores are small, coherent, granulated. At 11° to 12° C. no spores appear within a fortnight ; at 25° C., a very scanty spore-formation takes place in forty hours.

When the principal fermentation was broken off, the liquid contained 2.49 per cent. by vol. of alcohol ; in the following two periods 0.86 and 0.12 per cent. by vol. were formed.

Acid-production : 5.2.

Agreeable smell and fine aromatic taste.

6. (*Fig. 66, b, and Fig. 67, b.*)

The cells are round, oval, and elongated during fermentation, all forms occurring in chains ; isolated round giant cells occur.

The yeast lies rather compact in flask ; it requires strong shaking to distribute the cells equally throughout the liquid.

Film-formation : After 26 days the surface growth forms a ring of yeast cells on the wall of the flask ; only slight indications of film-formation. After 31 to 32 days the film had not developed further.

The cells of the ring-growth cannot be distinguished from those occurring during alcoholic fermentation.

If developed at a low temperature, the spores are comparatively small, granulated, with no distinct vacuoles. At 11° to 12° C., for seven days, very few spores are formed ; at 25° C., for forty hours, a scanty spore-formation takes place.

When the principal fermentation was broken off, the liquid contained 1.85 per cent. by vol. of alcohol ; during the following two periods 0.65 and 0.2 per cent. by vol. were formed.

Acid-production : 6.

Agreeable smell and slightly aromatic taste.

7. (*Fig. 66, c, and Fig. 67, c.*)

During fermentation round and oval cells, some free, others linked in short chains.

The yeast lies rather compact in flask ; violent shaking is required to distribute the cells equally throughout the liquid.

**Film-formation :** After 26 days a thin, almost continuous film appears, which in the course of the next five to six days forms a conspicuous covering extending over the whole surface of the liquid.

The cells of the film have in the main the same shape as those seen during fermentation ; only the youngest generations are elongated and narrow.

If developed at a low temperature, the spores are medium sized, with no distinct vacuoles. At 11° to 12° C., after nine days, fully developed spores appear ; at 25° C., for forty hours, spores are formed freely.

When the principal fermentation was broken off, the liquid contained 2·4 per cent. by vol. of alcohol ; during the following two periods 0·95 and 0·00 per cent. by vol. were formed.

**Acid-production :** 6·5.

Agreeable smell and slightly aromatic taste.

#### 8. (*Fig. 66, d, and Fig. 67, d.*) .

During fermentation, round, oval, and elongated cells, both free and linked together.

The yeast lies rather compact in flask ; on violent shaking the cells are distributed equally throughout the liquid.

**Film-formation :** After 31 to 32 days very slight isolated patches of a film on the surface, and a slight ring of yeast-cells on the glass, round the edge of the liquid.

The cells of the film have assumed quite different shapes from those of the fermentation-stage ; they are very much lengthened, mycelial, and irregular.

If developed at a low temperature, the spores are medium-sized, with no distinct vacuoles. At 11° to 12° C. spores are formed pretty freely on the ninth day ; at 25° C., they are formed freely in forty hours.

When the principal fermentation was broken off, the liquid contained 2·77 per cent. by vol. of alcohol ; during the following two periods 0·98 and 0·00 per cent. by vol. were formed.

Acid-production : 6.5.

Odour good, but bitter, persistent taste.

9. (*Fig. 66, e, and Fig. 67, e.*)

During fermentation a very uniform growth of big, round and oval cells.

The yeast lies rather loose in flask ; on shaking, the cells are distributed equally throughout the liquid.

Film-formation : In 31 to 32 days very slight, isolated patches on the surface, and a slender ring of yeast cells on the glass, round the edge of the liquid.

The cells of the film differ but little from those of the fermentation.

If developed at a low temperature, the spores are medium in size and granulated. At 11° to 12° C. spore-formation sets in on the ninth day ; at 25° C., for forty hours, a somewhat scanty spore-formation takes place, accompanied by a considerable formation of net-work.

When the principal fermentation was broken off, the liquid contained 2.96 per cent. by vol. of alcohol ; during the following two periods 1.19 and 0.00 per cent. by vol. were formed.

Acid-production : 7.

Odour good, pronounced vinous taste.

Regensburger has since undertaken very detailed comparative experiments with regard to three species of top-fermentation yeasts, which, like the examples just referred to, display characteristic differences in the general appearance of the young growths. Spore-formation occurs within differing periods, and conforms to the rule laid down by the author many years ago that the development of spores usually takes place more rapidly with top yeasts than with bottom yeasts. Distinct points of difference can also be observed in the visible course of fermentation, in the cardinal points for skin formation and in the development on solid substrata.

At the time, the author's argument that purely-cultivated top yeasts would quickly become impure on account of the prevailing high temperatures was subjected to criticism. Experience has shown that these criticisms have no weight,

and that great progress may be made in this field, and considerable advantages may be derived by the use of a single selected type. A further objection raised was that it is impossible by means of a single species to obtain a stable secondary fermentation, a wrong assumption previously made regarding a low-fermentation yeast. Van Laer strongly emphasised this view, and while he freely admitted that low yeast types exist, capable of carrying through a normal secondary fermentation, he believed that the author was wrong in ascribing the same properties to top yeasts. Notwithstanding the practical results attained by exact experiments carried out with selected types, even when due allowance was made for the special English conditions referred to by van Laer, and in spite of the fact that no exact proof was forthcoming to warrant the opposite view, the author's experience was ignored, and van Laer prepared mixtures of top-yeast species which were distributed for use in breweries. They were designed to satisfy practical demands, the intention being that one species should carry through the primary fermentation, the other the secondary fermentation. It is true that the possibility is not excluded that such a composite yeast could be prepared, but even when van Laer's preparations gave good results in practice it could not be proved that it was due to the activity of the composite yeast as such. It must first be demonstrated that this new yeast really reacted as a composite yeast—*i.e.*, that the different constituents are really capable of acting together. In conjunction with J. C. Holm, the author investigated many of the preparations distributed in the industry, and it was shown that even during the primary fermentation one of the species very strongly predominated, whilst in the secondary fermentation the other species disappeared. Thus the problem of preparing a truly composite yeast had not been solved. The experience of subsequent years has always confirmed the correctness of the first results, even in fermentations carried out on the English system. It is possible in both top and bottom fermentations to carry out the whole primary and secondary fermentation with a single selected species.

Quite recently the question of applying two species in English top-fermentation has been re-opened. It has been

supposed that the secondary fermentation of stout is brought about by certain species of *Torula* (two are described in the systematic part of this chapter), and that it is due to their activity that this variety of beer acquires its peculiar sourish taste. Here, however, two separate facts have been confused. The true secondary fermentation is carried out by the *properly selected type* of yeast, and can be regulated like any other secondary fermentation. In this connection the activity of the *Torulas* is unnecessary, as may plainly be seen from the fact that in every European country, and in other parts of the world, as shown by the author, stout and similar kinds of beer can be prepared from one of certain selected types of yeast. These species of *Torula* are to be reckoned in the same category as the lactic-acid bacteria, acetic-acid bacteria, *Sarcina*, etc., which also impart a special taste to a fermented wort. If such a taste is desired, it is of importance to regulate the activity of the organism in question, so that the quantity of the peculiar fermentation or assimilation product may stop short of a given limit. If this is exceeded the liquid becomes undrinkable.

It has frequently been suggested within recent times that all the races of culture yeast present in an impure top-fermentation yeast should be isolated and then again mixed in the same proportion, with a view of using this culture mixture in a brewery, where it would reproduce the original yeast, freed from bacteria and wild yeasts. Such a process, however, could not be carried out, at any rate, in this world.

After the author had introduced pure cultures into practice in many European countries, the reform met with general agreement. The work was taken up in the early stages by Kokosinsky, de Bavay, M'Cartie, W. R. Wilson, A. Miller, and R. Grey, to give but a few names. At a later stage, J. Schönfeld attacked the problem, and selected types of top yeasts were introduced into many German breweries, though chiefly as pitching yeast and not as pure culture.

In the case of top-fermentation lactic-acid beers, like German "Weissbier," the rational treatment must consist in the main in first carrying through a lactic-acid fermentation, and then applying the pure-cultivated top yeast.

**(b) Distillery and Pressed Yeasts.**

To solve the problem whether distillery and pressed yeasts are capable of forming endogenous spores, a possibility denied by Wiesner and Brefeld, the author, in 1884, undertook an exact examination of a number of samples of such yeasts, and in the same year, together with Hansen, published the results in Dingler's *Polytechnic Journal*, showing that there is no possible difficulty in obtaining an abundant and rapid spore-formation from these species. At the same time the author was able to arrive at certain conclusions regarding the composition of such yeast. By the help of fractional cultures, it was found that both top and bottom fermentation species occur in ordinary distillery and pressed yeast. Further investigations showed that in one and the same mass of yeast two morphologically different types may frequently occur, one chiefly giving isolated cells in a fully-developed state, the other, budding colonies of many cells. It was impossible, therefore, to trace any connection between this and the fermentation phenomena brought about by the two species. The two morphological types remain unaltered after being preserved for years.

Detailed researches further showed that both pressed yeast and the top yeast used in distilleries include a multitude of clearly distinguishable types, and a few years later pure selected races from the author's laboratory were first introduced into yeast factories, and then into the distilleries of Northern Europe and into Molasses factories.

Owing to the physiological state of the species, due to the dissimilar composition of the nutritive fluids, very important differences are exhibited with respect to propagating power, yield of alcohol, character of the alcohol, etc. These are retained throughout many years, so that it is necessary in many cases to instal an absolutely pure culture of a suitable yeast in each individual factory. By expert application of such cultures, and particularly by a rational lactic acidification, which arrests the development of foreign organisms found in the mash, it is possible to secure a higher yield and a better quality both of alcohol and of yeast. Rayman

and Kruis have undertaken elaborate investigations with regard to the character of the distillate obtained by the use of different species.

In 1890 the Berlin Experimental Station sent out the first yeast species cultivated from this group by P. Lindner. It was described as *Race 2*. More recently another species, *Race 12*, has been brought into practical use, and this appears to be preferred, according to Lindner's communications, both in potato distilleries and in pressed yeast factories. The yeast is grown in ordinary large fermenting vats, and is supplied in a pressed state in kilogramme lots.

Henneberg has given a detailed description of both species, from which it may be noted that the giant colonies differ in appearance. In *Race 2* they have an almost smooth surface, scored by a few shallow, concentric and radial furrows; the outline is fairly straight. *Race 12* has a very uneven surface, scored by deep, irregularly radial furrows. The ridges so formed constitute an extremely delicate concentric pattern, and the outline is formed erratically by the termination of the ridges at varying distances from the centre. The small colonies in plate-cultures are similar. *Race 2* has feebly-developed budding colonies, whilst *Race 12* forms large and dense clusters. The cells of *2* are an elongated oval; those of *12* are roundish and oval.

#### (c) Wine Yeasts.

When a number of pure growths are isolated from the usual elliptical wine yeasts, it will be readily seen that they vary greatly in morphological character, under similar conditions of cultivation, especially if the general appearance is taken into account. Species with both large and small cells and every intermediate form are met with, from elongated and elliptical, to oval and almost spherical cells. *Pastorianus* forms of yeast also exist. Before 1890 a series of such types, exhibiting stable morphological characters, and displaying characteristic differences in spore-formation, had been isolated in the author's institute.

Hansen had published further information regarding the individual species in his description of *S. ellipsoides* I. (see

following section), and subsequently in his notes regarding *Johannisberg II*. He found that the temperature limits for budding in wort are 37° to 38° C. and 0.5° C.; for spore-formation on gypsum blocks 33° to 34.5° C. and 2° to 3° C. Further publications we owe to Aderhold, Hotter, Kayser, Marx, Müller-Thurgau, Seifert, Wortmann, and others. As examples of the different biological characters observed in wine yeast, we will discuss more closely a few of the species described by Aderhold.

*Johannisberg I.* has round or oval-pointed cells; in the young film produced at 26° to 27° C.\* the cells are oval; spores appear in 28 to 30 hours at 25° to 26.5° C.\*

*Johannisberg II.* has large oval cells, characterised by longish but blunt ends. The film cells are round, oval, and sausage-shaped; spores are formed within 23 to 24 hours.

*Kreuznach* has the same cell-formation as the previous species, but somewhat smaller; film cells like *Johannisberg I.*; spores in 30 hours.

*Mülheim* has broad, oval, and, less frequently, round cells, with short pointed ends smaller than the previous species; only round and oval film cells; spores within two to three days.

*Walporzheim I.* has round cells, the oval forms scarcely pointed; often budding colonies in the film, elongated links forming an axis for the colony surrounded by round cells; spores in 80 hours.

*Piesport*; predominantly elliptical cells without pointed ends; only spherical cells in the film; spores in 23 to 24 hours. Grown on solid substrata, differences can be observed in the development of colonies.

Pure selected races have gradually been introduced in large numbers into the wine fermentation by Müller-Thurgau, Wortmann, Kayser, Jacquemin, the author, and others. Müller-Thurgau and Wortmann, amongst others, have indicated and proved the importance of these pure cultures, as not only the must but also the fermented product, the wine, is dominated by the pure yeast throughout every stage of its development, extending, it may be, through many years.†

\* Similar temperatures hold good for the following species.

† See the section on the behaviour of *Saccharomyces* with sugars, etc.

## 2. YEASTS NOT YET APPLIED INDUSTRIALLY.

**Saccharomyces Pastorianus** or **Saccharomyces Pastorianus I.**  
Hansen (Figs. 68, 69).

Bottom-fermentation yeast.

Sedimentary forms developed in yeast:—Predominantly elongated and sausage-shaped, also large and small oval and round cells (Fig. 68). When this species is cultivated in wort near its maximum temperature for growth, its vegetation consists of sausage-shaped and elongated cells. The temperature limits for budding in wort are 34° and 0·5° C.

It frequently occurs in the air of fermenting rooms, and is also found in diseased beers. It imparts to beer a disagreeable bitter taste and unpleasant odour; it may also produce turbidity, and interfere with the clarification of beer in the fermenting vessel.

According to the investigations of Mach and Portele, this species may also be successfully used in wine fermentation. Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose.

Ascospore-formation (Fig. 58, 2):—

At 31·5° C. no ascospores are developed.

29·5-30·5 the first indications are seen after 30 hours.

29	„	„	27	„
27·5	„	„	24	„
23·5	„	„	26	„
18	„	„	35	„
15	„	„	50	„
10	„	„	89	„
8·5	„	„	5 days.	
7	„	„	7	„
3·4	„	„	14	„
0·5	no ascospores are developed.			

Size of spores 1·5 to 5  $\mu$ .

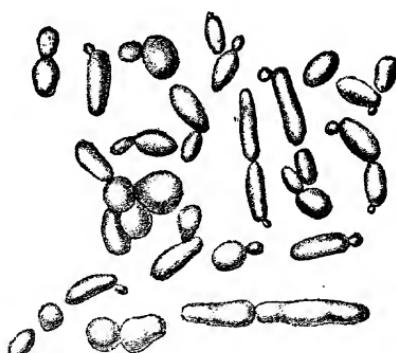


Fig. 68.—*Saccharomyces Pastorianus I.* (Hansen).—Cell-forms of young sedimentary yeast (after Hansen).

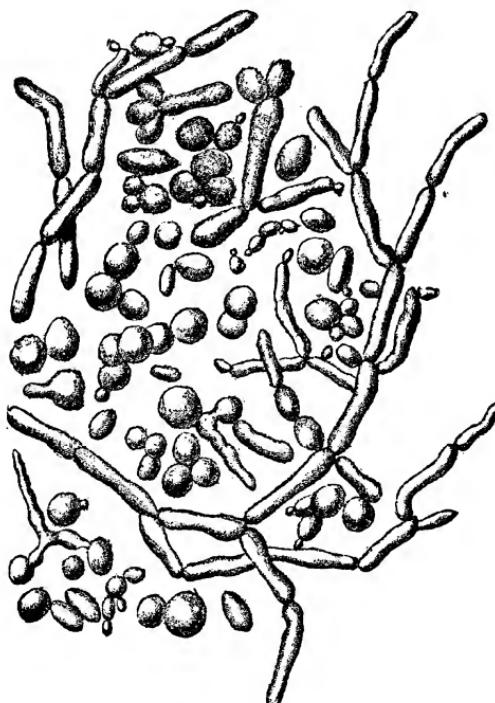


Fig. 69.—*Saccharomyces Pastorianus I.* (Hansen).—Film-forms at 13° to 15° C., from Holm's drawing in Hansen's Memoir.

## Film-formation :—

At  $34^{\circ}$  C. no film-formation occurs.

26-28 feebly-developed film-specks

are seen after . . . 7-10 days.

20-22 " " 8-15 "

13-15 " " 15-30 " ) (Fig. 69.)

6- 7 " " 1- 2 months, ) (Fig. 69.)

3- 5 " " 5- 6 "

like Fig. 69, but without the large colonies.

2- 3 no film-formation occurs.

## Microscopic appearance of the cells in the films :—

At  $20^{\circ}$  to  $28^{\circ}$  C. almost the same forms occur as in the sedimentary yeast.

At  $13^{\circ}$  to  $15^{\circ}$  C. strongly-developed mycelial colonies of very elongated, sausage-shaped cells are fairly frequent (Fig. 69).

In old cultures of films the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

*Saccharomyces intermedius* or *Saccharomyces Pastorianus* II.

Hansen. (Figs. 70, 71.)

Feeble top-fermentation yeast.

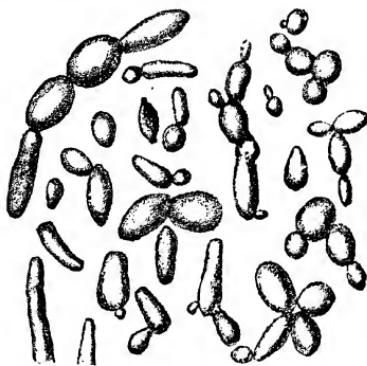


Fig. 70.—*Saccharomyces Pastorianus* II. (Hansen).—Cell-forms of young sedimentary yeast  
(after Hansen).

Sedimentary forms grown in wort :—Mainly elongated, sausage-shaped cells, but also large and small, oval, and round cells (Fig. 70).

When this species is cultivated in wort near the maximum temperature for growth its vegetation consists of round and oval cells. The temperature limits for budding in wort are 40° and 0.5° C.

It frequently occurred in Hansen's analyses of air in the brewery; it appears to belong to the species which do not cause diseases in beer. Saccharose, dextrose, laevulose, and maltose are fermented, but not lactose.

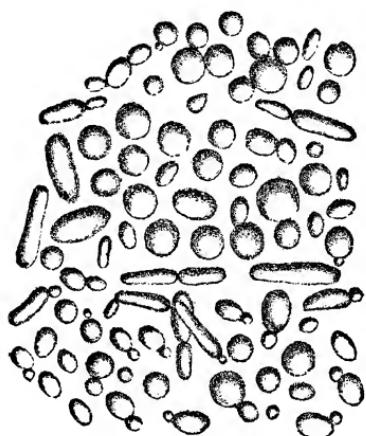


Fig. 71.—*Saccharomyces Pastorianus II.* (Hansen).—Film-forms at 15° to 3° C. (after Holm's drawing in Hansen's Memoir).

#### Ascospore formation (Fig. 58, 3):—

At 29° C. no ascospores are developed.

27-28 the first indications are seen after 34 hours.

25	"	"	25	"
23	"	"	27	"
17	"	"	36	"
15	"	"	48	"
11.5	"	"	77	"
7	"	"	7 days.	
3-4	"	"	17	"

0.5 no ascospores are developed.

Size of the spores 2 to 5  $\mu$ .

## Film-formation :—

At	34° C.	no film-formation occurs.
26-28	feebley-developed film-specks	are seen, after . 7-10 days.
20-22	„ „ „	8-15 „
13-15	„ „ „	10-25 „
6- 7	„ „ „	1- 2 months. } (Fig. 71.)
3- 5	„ „ „	5- 6 „
2- 3	no film-formation occurs.	

## Microscopic appearance of the cells in the films :—

At 20° to 28° C., almost the same forms as in the sedimentary yeast ; also irregular sausage-shaped cells.

At 15° to 3° C., mostly oval and round cells.

In old cultures of films the cells are smaller than in the sediment ; very irregular and sometimes almost thread-like cells are found.

Streak cultures of this species in yeast-water gelatine give growths with comparatively smooth edges after sixteen days at 15° C., and in this respect it also differs from the following species.

*Saccharomyces validus* or *Saccharomyces Pastorianus* III.  
Hansen. (Figs. 72, 73.)

## Top-fermentation yeast.

Sedimentary forms grown in wort :—Mostly elongated, sausage-shaped, but also large and small, oval, and round cells (Fig. 72). When this species is cultivated in wort near the temperature maximum for growth, the vegetation consists of round and oval cells. The temperature limits for budding in wort are 39° to 40° C. and 0-5° C.

It was separated from a bottom-fermentation beer which showed yeast-turbidity, and has been proved by Hansen to be one of the species which produce this disease. Recent experiments of Hansen show that this disease-yeast possesses another peculiar property—its addition will in certain cases effect a clarification when the fermenting wort has an opalescent appearance.

According to investigations made by the author, a strong infection of low-fermentation yeast with this species may in

certain cases effect an excellent clarification and good "breaking" in both fermentation vessel and cask. Saccharose, dextrose, laevulose, and maltose are fermented, but not lactose.

Ascospore-formation (Fig. 58, 4) :—

At 29° C. no ascospores are developed.

27-28 the first indications are seen after 35 hours.

26.5	"	"	30	"
25	"	"	28	"
22	"	"	29	"
17	"	"	44	"
16	"	"	53	"
10.5	"	"	7 days.	
8.5	"	"	9	"

4 no ascospores are developed.

Size of the spores 2 to 5  $\mu$ .

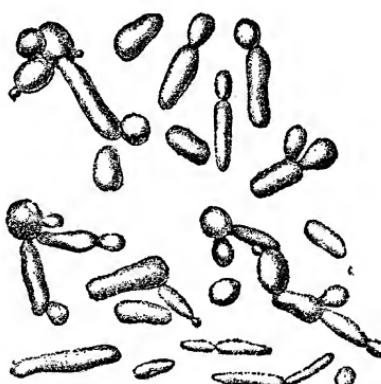


Fig. 72.—*Saccharomyces Pastorianus III.* (Hansen).—Cell-forms of young sedimentary yeast (after Hansen).

Film-formation :—

At 34° C. no film-formation occurs.

26-28 feebly-developed film-specks

are seen after . . . 7-10 days.

20-22	"	"	9-12	"
13-15	"	"	10-20	"
6-7	"	"	1-2 months.	
3-5	"	"	5-6	"
2-3	no film-formation occurs.			

} (Fig. 73.)

Microscopic appearance of the cells in the films :—

At 20° to 28° C. : Almost the same forms as in the sedimentary yeast.

At 15° to 3° C. : Strongly-developed colonies of elongated, sausage-shaped or thread-like cells, which closely resemble a mycelium in appearance (Fig. 73).

In old cultures of films, the cells have the same forms as at 15° to 3° C., but are often still thinner and more thread-like.

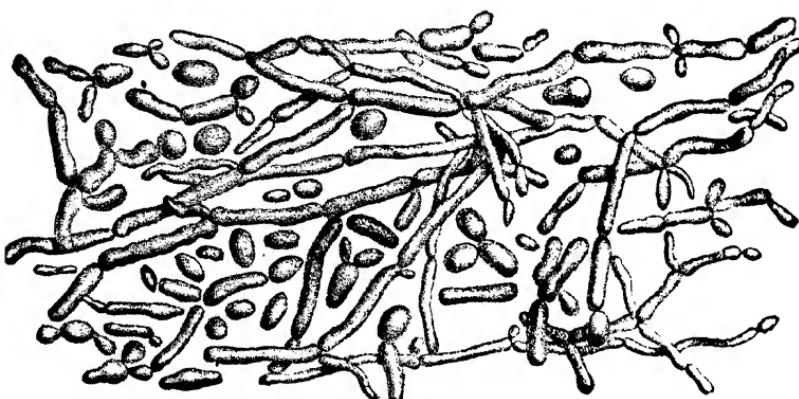


Fig. 73.—*Saccharomyces Pastorianus III.* (Hansen).—Film-forms at 15° to 3° C. (after Hansen).

Streak cultures of this species in yeast-water gelatine, after sixteen days at 15° C., give growths with distinctly hairy outline.

***Saccharomyces ellipsoideus* or *Saccharomyces ellipsoideus* I.  
Hansen. (Figs. 74, 75.)**

Bottom-fermentation yeast.

Sedimentary forms grown in wort :—Mostly oval and round cells ; sausage-shaped cells are rare (Fig. 74).

If this species is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells. The temperature limits for budding in wort are 40° to 41° C. and 0.5° C. Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose.

Occurs on the surface of ripe grapes.

## Ascospore-formation (Fig. 58, 5) :—

At  $32.5^{\circ}$  C. no ascospores are developed.

30.5-31.5 the first indications are seen after 36 hours.

29.5	„	„	23	„
25	„	„	21	„
18	„	„	33	„
15	„	„	45	„
10.5	„	„	$4\frac{1}{2}$	days.
7.5	„	„	11	„
4	no ascospores are developed.			

Size of the spores 2 to  $4 \mu$ .

## Film-formation :—

At  $38^{\circ}$  C. no film-formation occurs.

33-34 feebly-developed film-specks

are seen after 8-12 days.

26-28	„	„	9-16	„
20-22	„	„	10-17	„
13-15	„	„	15-30	„, (Fig. 75.)
6-7	„	„	2-3 months.	
5	no film-formation occurs.			

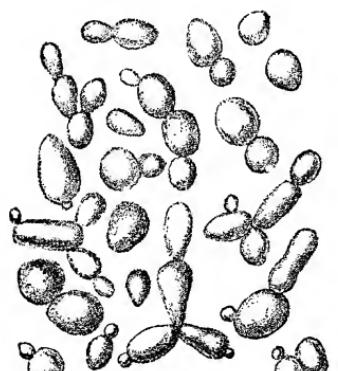


Fig. 74.—*Saccharomyces ellipsoideus* L. (Hansen).—Cell-forms of young sedimentary yeast (after Hansen).

## Microscopic appearance of the cells in the films :—

At  $20^{\circ}$  to  $34^{\circ}$  C. and  $6^{\circ}$  to  $7^{\circ}$  C., the cells are smaller and more sausage-shaped than in the sedimentary yeast.

At  $13^{\circ}$  to  $15^{\circ}$  C., freely-branched and strongly-developed colonies of long or short sausage-shaped cells, often with verticillated branches (Fig. 75).

In old cultures of films, the cell forms are the same as at  $13^{\circ}$  to  $15^{\circ}$  C.

Streak cultures of this species in wort-gelatine (wort with the addition of about 5.5 per cent. of gelatine), in the course

of eleven to fourteen days at 25° C., give—in contradistinction to the other five species—a characteristic net-like structure, by

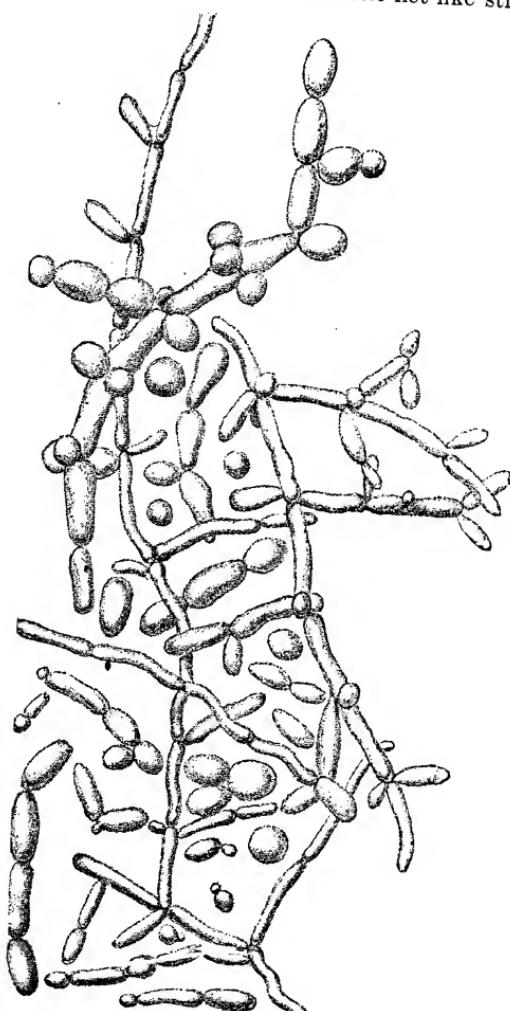


Fig. 75.—*Saccharomyces ellipsoideus* I. Hansen.—Film-forms at 13° to 15° C. (from Holm's drawing in Hansen's Memoir).

means of which it can be distinguished by the naked eye from other species.

**Saccharomyces turbidans or Saccharomyces ellipsoideus II.  
Hansen. (Figs. 76, 77.)**

Usually a bottom-fermentation yeast.

Sedimentary forms grown in wort :—Oval and round cells predominate ; sausage-shaped cells are rare (Fig. 76).

It was separated from beers which showed yeast-turbidity ; is a species which causes yeast-turbidity, and is more dangerous than *Sacch. Pastorianus III.* If this species is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells. The temperature limits for budding in wort are 40° C. and 0.5° C. Saccharose, dextrose, levulose, and maltose are fermented, but not lactose.



Fig. 76.—*Saccharomyces ellipsoideus II.* Hansen.—Cell-forms of young sedimentary yeast (after Hansen).



Fig. 77.—*Saccharomyces ellipsoideus II.* Hansen.—Film-forms at 28° to 3° (after Hansen).

Ascospore-formation (Fig. 58, 6) :—

At 35° C. no ascospores are developed.

33-34 the first indications are seen after 31 hours.

33	„	„	27	„
31.5	„	„	23	„
29	„	„	22	„
25	„	„	27	„
18	„	„	42	„
11	„	„	5½	days.
8	„	„	9	„
4	no ascospores are developed.			

Size of spores 2 to 5  $\mu$ .

## Film-formation :—

At  $40^{\circ}$  C. no film-formation occurs.

36-38 feebly-developed film-specks  
are seen after . 8-12 days.

33-34        „        „        3- 4    „

26-28        „        „        4- 5    „

20-22        „        „        4- 6    „

13-15        „        „        8-10    „

6- 7        „        „        1- 2 months.

3- 5        „        „        5- 6    „

2- 3        no film-formation occurs.

} (Fig. 77.)

## Microscopic appearance of the cells in the films :—

At all temperatures, the same forms as in the sediment; at and below  $15^{\circ}$  C., the cells are only slightly more elongated (Fig. 77).

*Saccharomyces Willianus Saccardo*

is a disease yeast described by Will as having elliptical cells. In the old films strongly branched budding-colonies occur consisting of very long cells. The maximum temperature for spore-formation is  $39^{\circ}$  C.; at the optimum temperature ( $34^{\circ}$  C.) the first traces of spores occur in eleven hours. The lowest limit for spore-formation is  $4^{\circ}$  to  $5^{\circ}$  C. The limits for film-formation are  $41^{\circ}$  and  $4^{\circ}$  C. The vegetative cells are killed when heated in sterilised wort for half-an-hour at  $70^{\circ}$  C. It forms colonies in wort gelatine, which in the young state (both embedded and on the surface) form a network of wide mesh. They afterwards become denser in the middle with irregularly fringed edges. Sometimes, however, under the same conditions, compact colonies with regular outline are formed.

This species imparts a peculiar sweet taste to beer, followed by a rough bitter after-taste, even in presence of very minute quantities (0.1 per cent.) in the pitching yeast. The beer is often rendered turbid by this wild yeast after two months at  $4^{\circ}$  to  $5^{\circ}$  C. This yeast has a strong fermenting and propagating power, and is very dangerous for beer.

**Saccharomyces Bayanus Saccardo**

was discovered by Will in turbid beer. The cells are elliptical. In old films strongly branching budding-colonies occur. The temperature limits for spore-formation are 32° and 0.5° C.; the optimum is 24° C. The limit for existence of the vegetative cells in wort is 70° C.

Besides causing yeast turbidity this species also imparts a sweetish, disagreeable, aromatic taste to beer, and an unusually bitter herbal and astringent after-taste. The odour is aromatic, like that of rotten fruit. With mixtures of about 29 per cent. of the wild yeast the flavour is very strongly pronounced, and may be recognised even in the presence of 5 per cent. The yeast causes a discolouration of beer. It turns paler, and assumes a foxy appearance.

**Saccharomyces Logos Van Laer**

was derived from a yeast in Logos & Co.'s brewery in Rio de Janeiro, and is a bottom-fermentation yeast of a *Pastorianus* shape, which occurs in the fermenting vessel as a loosely-lying sedimentary yeast of cheesy appearance; consequently the beer clarifies very rapidly. The fermentation is carried out at high temperatures (22° to 30° C.). The yeast ferments very slowly, but gradually produces very high percentages of alcohol. The flavour of the beer is entirely different from that of ordinary lager beer. According to Rothenbach this species is able to ferment about half of a diastase-dextrin prepared according to Lintner's recipe, but is distinguished from *Schizosaccharomyces Pombe* by fermenting other kinds of dextrin. According to Prior and Weigmann, it ferments Achroodextrin III. (Prior) completely, whilst Achroodextrin II. is only fermented to the extent of 75 per cent.

Meissner determined the action of different acids on the Logos yeast (in comparison with Saaz and Frohberg) using a nutritive liquid consisting of 10 per cent. cane-sugar solution with 10 per cent. of yeast-water. The fermentative activity of Logos yeast was increased at first on the addition of 0.375 per cent. of acetic acid (the other two yeasts with 0.25 per cent.). A smaller quantity of acetic acid (0.125 per cent.)

caused the Logos yeast to bring about inversion and to begin fermentation more rapidly. It can also withstand larger quantities of lactic acid than the other two species. In the presence of lactic acid the formation of alcohol is reduced, but by the addition of 0.125 per cent. the Logos yeast gives more acid than usual. In general the production of acid is considerably greater in the presence of lactic than in the presence of acetic acid. In the presence of both acetic and lactic acid the formation of volatile acids is considerably greater than that of non-volatile. The fermentative energy (four days) is greater in presence of acetic acid than in the absence of acid, but the addition of lactic acid considerably restricts it.

According to Korff, non-volatile acids are formed in larger quantities in aerated cultures; on the other hand, more volatile acids are formed when hydrogen is passed through the solution.

According to Bau, Logos yeast does not contain melibiase, whereas Lindner arrived at the opposite result. Further research has shown that both authors are right, for a similar subdivision of the race takes place as in the case of *Torula colliculosa* (see below).

#### *Saccharomyces thermantitonum* Johnson

is a yeast found on eucalyptus leaves, and was accidentally grown in a flask which had been infected at a temperature of 84° C. The cells had not, however, been killed at this high temperature. By subsequent repeated observations, it was found that this species reacts best at high temperatures. Johnson used a temperature of 50° C. as the pitching temperature for fermentations on the large scale. The wort need not, therefore, be cooled so strongly as usual, and the yeast can be applied in tropical countries where ice machines are not available. Within seventeen hours it is said to furnish a properly and completely fermented beer. It does not grow at temperatures under 10° C., and its optimum temperature for propagation and fermentation lies between 40° and 44° C. The cells are small and oval. They agglutinate and form clots, which sink rapidly and adhere closely to the bottom. Consequently, the liquid clarifies very rapidly.

**Saccharomyces Ilicis Grönlund**

which was found on the fruit of *Ilex Aquifolium*, is a bottom-fermentation yeast, consisting mainly of spherical cells. The temperature limits of spore-formation are 38° and 8° C. The spores have no vacuoles. In the films slightly-elongated cells are found. Streak cultures on gelatine have a floury, but otherwise variable, appearance. This species, grown in wort, imparts a disagreeable, bitter taste. According to Schjerning, it contains invertase, and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can yield about 2.8 per cent. of alcohol (by volume).

**Saccharomyces Aquifolii Grönlund**

was also found on the fruit of *Ilex Aquifolium*. It is a top-fermentation yeast, and consists of large round cells. The temperature limits for spore-formation are 31° and 8° C. ; the spores contain vacuoles. In the films, spherical and egg-shaped cells alone occur. Streak cultures in gelatine vary in appearance, some being glossy and some floury. This species imparts to wort a disagreeable, sweet taste, with a bitter after-taste. It inverts saccharose and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can yield about 3.7 per cent. of alcohol (by volume).

**Saccharomyces pyriformis Marshall Ward**

(see Ginger-beer Plant).

**Saccharomyces Vordermanni Went and Prinsen-Geerligs**

was discovered in Java as an essential factor in the manufacture of arrack. It is distinguished by its powerful action as an alcoholic ferment, and yields a fine distillate on account of which it is made use of, in pure culture, in the manufacture of arrack. The cells are ellipsoidal, and form not more than four spores. This species ferments dextrose, maltose, and saccharose, and contains invertase.

**Saccharomyces Saké Yabe.**

Y. Kozai has published the following conclusions regarding this yeast fungus. He discovered it in Koji, and has utilised it with success in a pure condition for the preparation of Saké. The cells are chiefly round, and form no large budding colonies. In older cultures giant cells occur. Indications of spores occur within 36 hours at 40° to 41° C. (maximum temperature), in 14 hours at 30° to 32° C. (optimum temperature), and in 15 days at 3° to 4° C. (minimum temperature). The spores (usually from one to three in each cell) are strongly refractive. It ferments with ease saccharose, maltose, *d*-mannose, *d*-fructose, glucose, and methyl-glucoside, and with greater difficulty trehalose and *d*-galactose, but not rhamnose nor lactose. It splits up melitriose into melibiose and fructose, but it cannot hydrolyse melibiose. K. Yabe's investigations proved that rice straw is the source of the yeast, a straw used for the preparation of mats which serve to cover up the Koji.

**Saccharomyces Batatae Saito.**

K. Saito has described a *Saccharomycetes* which is of importance in the preparation of a yam brandy, as it is prepared on one of the Japanese islands. The species is found in fermenting mash (moromi) prepared from Koji and steamed yams, and is the most active organism in promoting alcoholic fermentation. The cells are oval and elliptical, and *Pastorianus* forms often occur in the film. Indications of spores occur in twenty hours at 25° C. The spores are round, strongly refractive, and usually occur two to three in each cell. In ordinary beer-wort 3 per cent. by volume of alcohol was produced in ten days at 25° C.

Dextrose, lævulose, saccharose, and maltose are easily fermented; galactose and raffinose with difficulty; and melibiose, lactose, inulin, and *d*-methylglucoside are not fermented.

**Saccharomyces cartilaginosus Lindner**

was discovered by Matthes in Kephir. The cells are oval or elongated, with curious granular protoplasm. A true film-formation does not take place. On the other hand, at the

completion of the fermentation, clearly isolated islands of somewhat dense and almost cartilaginous consistency appear on the surface of the wort. It ferments saccharose and maltose, but not lactose.

**Saccharomyces multisporus n.sp.**

is an elliptical wild yeast which was found in a few cases in English top-fermentation yeasts by J. C. Holm. Many very large round cells (giant cells) are found amongst the elliptical, within which nine to eleven spores may form, whilst the elliptical cells occur with two to four spores. The spores are round and strongly refractive. At 25° C. ripe spores are formed in forty hours, at 15° C. in seventy-two hours. The yeast is a bottom-fermentation species, and adheres so closely to the flasks that it can hardly be loosened by shaking. It forms as a thin film, which first covers the bottom, and then the sides of the flask. In ordinary wort it yields about 4 per cent. by weight of alcohol. The taste is strongly bitter. It ferments dextrose, maltose, and saccharose. By preservation both in saccharose and in wort, the giant cells lose their power to produce such an exceptionally large number of spores; in gypsum-cultures most of these cells did not develop spores, but many vacuoles.

**Saccharomyces mali Risler Kayser**

is found in cider, and imparts to it a uniform flavour. The cells are round, and it does not form a film. At 15° C. spores appear in 96 hours. It is a bottom yeast, which ferments saccharose, dextrose, and maltose. The sedimentary yeast adheres closely to the flask.

---

**Saccharomyces Marxianus Hansen.**

This species, which was discovered by Marx on grapes, and described by Hansen, develops in beer-wort in the form of small oval cells, similar to those of *Sacch. exiguum* and *ellipsoideus*. Elongated, sausage-shaped cells, often in colonies, soon appear, however, and if the culture is allowed to stand for some time, small mould-like particles are formed; some of

these swim in the liquid, others settle to the bottom. These particles consist of mycelial colonies of practically the same character as the film-formations of Hansen's six species ; they are also built up of cells, which are readily separated at the point of union. When *S. Maxxianus* is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells. The ascospores are kidney-shaped, spherical, or oval. After cultivation for two to three months in wort contained in two-necked flasks, there were only traces of film-formation with few sausage-shaped and oval cells.

This yeast is one of those species which develop a mycelium under certain conditions of culture on a yeast-water gelatine.

In beer-wort it yields only 1 to 1.3 per cent. by volume of alcohol, even after long standing. It does not ferment maltose ; it inverts saccharose ; and in nutritive solutions of the latter, and of dextrose, it yields considerable quantities of alcohol. The temperature limits for budding in wort are 46°-47° C. and 0.5° C.

The maximum temperature for spore-formation lies between 32° and 34° C., the minimum between 4° and 8° C., the optimum between 22° and 25° C. The growth yields quicker and more abundant spore-formation if cultivated in yeast-water, or wort with 10 per cent. dextrose (Klöcker).

In agreement with his theory that maltose is split up by a particular enzyme differing from invertase, and only subsequently fermented, Emil Fischer found that an aqueous extract of the pulverised growth of this yeast decomposed saccharose, but not maltose.

#### *Saccharomyces exiguum* (Reess) Hansen

develops a growth in wort, the cell-forms of which most closely correspond to the species described by Reess under the above name. It is, however, impossible to determine whether Reess was really dealing with this species, since any *Saccharomyces* species may, under certain conditions, form a preponderating number of similar small cells.

This species only gives scanty spore-formation and weak film-formation, but it yields a well-developed yeast ring. The

cells of the film resemble those of the sedimentary yeast, but short sausage-shaped and small cells are more frequent.

Hansen found this species in pressed yeast. Its behaviour towards the sugars is similar to that of the last species, though it develops a greater fermentative activity in solutions of saccharose and dextrose. Like the former species, it yields in wort only small quantities of alcohol. It does not ferment maltose solutions. It inverts saccharose.

Experiments, carried out by Hansen in practice, have shown that this species does not produce any disease in beer, even when present in considerable quantities either at the beginning or end of the primary fermentation, or when it is added after the storage of beer.

This is of special interest, as *Sacch. exiguis* was formerly regarded as a disease-producing species.

#### *Saccharomyces Jörgensenii Lasché*

also belongs to this group of the *Saccharomycetes*. The growth consists of small round and oval cells. The optimum temperature for spore-formation is 25° C., the temperature limits being 8° and 30° C. At temperatures above 30° C. the growth dies rapidly. A true film-formation has not been observed; in old cultures only a very feeble yeast ring forms, consisting of round and oval cells. In gelatine it yields colonies which resemble those of low-fermentation brewery yeast. Wort-gelatine is slowly liquefied. The streak-culture is a dirty grey colour, and has smooth edges. This species ferments saccharose and dextrose, but not maltose. Consequently it is suppressed when mixed with cultivated yeasts and grown in malt-wort. In wort it yields only 0.89 per cent. by weight of alcohol. In "temperance beer," according to Lasché's statement, it produced a strong turbidity.

#### *Saccharomyces Zopfii Artari*

was discovered by Zopf in the syrup of a sugar factory. The cells are small, spherical, or elliptical. The temperature maximum for budding in wort is 33° to 34° C., the optimum 28° to 29° C. The temperature maximum for spore-formation

is 32° C., optimum 26° to 29° C. The spores (usually two in each cell) are round. They are easily produced both in liquid and on solid substrata, and especially well in tartaric acid solution containing potassium nitrate. The fungus can supply its demand for carbon from saccharose, glucose, and mannite, but not from maltose, lactose, galactose, inulin, and melampyrite. It is capable of fermenting a saccharose solution containing 50 per cent. of sugar. During fermentation an acid is produced which at a later stage is used up.

According to Artari, it can satisfy its requirements for nitrogen with ammonium sulphate. When the species is cultivated in a dextrose solution containing from 5 to 8 per cent. of ammonium sulphate, transverse walls make their appearance between the mother and daughter cells, as is the case with *S. Ludwigi*. In such a solution only spherical cells occur. The addition of potassium nitrate brings about an alteration to pear-shaped cells.

#### **Saccharomyces Bailii Lindner**

was isolated from Jopen beer-wort which had a primary concentration of 58° to 54° Balling. The cells are large, thick-walled, and elongated, and assume irregular shapes in old cultures (like *Amœba*). The spores are strongly refractive. There is no film-formation. It ferments dextrose and saccharose, but not *d*-galactose and *d*-mannose. It gives a feeble fermentation in wort, and in old cultures the wort has a slight perfumed odour. It forms the main constituent of yeast in Jopen beer samples, and doubtless plays a part in the preparation of Danzig Jopen beer.

#### **Saccharomyces hyalosporus Lindner**

forms a thin film on wort, and produces no fermentation in the different kinds of sugar. The spores are round, and resemble glass beads carrying a lustrous granule in the centre. It forms spores in the film. The cells are oval, and sometimes rather elongated. They are often linked together in chains.

It was discovered by Lindner in a sample of beer from a propagating apparatus.

**Saccharomyces Rouxi Boutroux**

was isolated from fermenting fruit juices, and appears to have been found by Roux in dextrose. It displays a remarkable behaviour to the sugars. Like *S. Soya*, it immediately ferments dextrose and maltose, but does not ferment saccharose and lactose. The cells are small, round, or oval, and linked together in chains. One, two, and three spores occur in the cells, and are also found in the film cells. The film does not cover the whole surface of the liquid, but forms islands of yeast dotted over the surface.

**Saccharomyces Soya Saito**

in the Japanese Soja (Shoju), which contains 15 to 17 per cent. of sodium chloride. Saito found two different *Saccharomyces*, one of which, he affirms, has an aromatic effect on the Soja fermentation. *S. Soya* occurs in large quantities, and forms the second species. The cells are round or oval. The spores are formed in the yeast ring, and preferably in abnormally constructed cells. Saito did not succeed, however, in obtaining spores on gypsum blocks after the yeast had been cultivated in wort or in dextrose yeast-water. Judging from Saito's sketches, this yeast must be a *Zygosaccharomyces*.

It ferments glucose, maltose, levulose, *d*-galactose, and *d*-mannose, but not saccharose, lactose, melibiose, raffinose, inulin, or *a*-methylglucoside. According to Saito, it contains invertase in the form of an endoenzyme.

Associated with this species a yeast was found forming spores in the film. The cells are round or oval, and occasionally mycelial forms are noted. It forms a white floury film on koji decoction. Abundant quantities of carbon dioxide collect under the film, and the latter soon assumes a yellowish-brown colour, and displays grooved wrinkles. In beer-wort it gives a top-fermentation, but no film-formation. The spores are round, and, according to Saito's drawing, this must also be a kind of *Zygosaccharomyces*.

It ferments glucose, maltose, and levulose, but not lactose, galactose, saccharose, melibiose, raffinose, inulin, or *a*-methylglucoside.

**Saccharomyces mali Duclaux Kayser**

was discovered in cider, to which it imparts a good bouquet and body. The cells are oval, and film-formation takes place. At 15° C. spores are formed in 84 hours. It is a top-fermentation yeast, and ferments dextrose, but not saccharose, maltose, and lactose. The sedimentary yeast lies very loosely in the flask.

**Saccharomyces unisporus n.sp.**

was discovered by J. C. Holm in Dutch cream. As regards its behaviour to the sugars, it is most nearly allied to *S. mali Duclaux*, for it ferments dextrose, but not saccharose, maltose, and lactose. The cells are small and oval. *Pastorianus* forms are also found in old cultures. The spores are round and refractive. Only one fairly large spore is found in each cell. At 25° C. a few cells are found with ripe spores in 40 hours, and at 15° C. in 72 hours. No true film-formation occurs, but, on the other hand, a yeast ring is formed in old cultures.

**Saccharomyces flava lactis Krueger.**

A yeast cultivated from cheesy butter must be alluded to, which imparts a curious yellow colour, forms yellow colonies on gelatine, and a yellow film on milk. The cells are small, elliptical, and linked in chains. It ferments dextrose with difficulty, and lactose not at all. On slices of carrot it quickly forms spores. The most favourable temperature for its growth lies between 18° and 20° C. It grows better on slightly alkaline or neutral substrata than on acid. It quickly liquefies gelatine. It only produces colouring matter when it is in contact with air.

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**Levure de sel α**

was discovered by Kr. Höye in air analyses which were carried out along the coast of Norway (Bergen and Christiansund). He used wheat paste mixed with about 17 per cent. of sodium chloride as the substratum. The yeast is round, and forms only a single spore in each cell. It thrives best in

a fish broth containing 10 per cent. of sodium chloride. In a nutritive liquor which contains less than 3 per cent. of salt the growth ceases. The cells do not alter their round shape in nutritive liquids with varying quantities of salt. It produces no fermentation in apple juice.

#### ***Saccharomyces Hansenii* Zopf**

was discovered amongst the fungi of cotton-seed meal. It forms spherical spores of very minute diameter, which are developed singly or never with more than two in a mother cell. In fermentable saccharine solutions it produces no alcoholic fermentation, but crystals of calcium oxalate are observed in the sediment. Zopf found a similar formation in nutritive solutions containing galactose, grape-sugar, saccharose, lactose, maltose, dulcite, glycerine, and mannite.

#### ***Saccharomyces minor* Engel.**

The vegetative cells are completely spherical, measuring about  $6\ \mu$  in diameter, and are linked in chains or specks containing six to nine cells. The spore-forming cells measure 7 to  $8\ \mu$ , and contain from two to four spores of  $3\ \mu$  diameter.

Engel believes this organism to be the most active ferment in the fermentation of bread. The author has frequently found this minute spore-forming yeast in the sour dough of the Copenhagen bakeries.

#### ***Pichia membranæfaciens* or *Saccharomyces membranæfaciens* Hansen.**

This peculiar species, which occupies a special place amongst the *Saccharomycetes*, yields a strongly-developed light grey wrinkled film when grown in wort, which rapidly covers the whole surface of the liquid, and consists mainly of sausage-shaped and elongated oval cells; these have strongly-developed vacuoles, and a more or less empty appearance. Separating the colonies is an abundant admixture of air.

The limits of temperature for budding on wort are  $35^{\circ}$  to  $36^{\circ}$  C., and  $0.5^{\circ}$  C. When this species is cultivated near the limiting temperature, it occurs entirely as sedimentary yeast.

According to Seifert, it grows even in presence of 12.2 per cent. by volume of alcohol.

The spores are very abundantly developed, not only under the ordinary conditions of cultivation, but also in films. They are irregular in form, and, at the ordinary room-temperature, germinate in a Ranzier chamber in ten to nineteen hours.

On wort-gelatine, the cells form dull grey specks, often with a faint, reddish tinge, which are rounded, flat, wide-spread, and wrinkled. The colonies embedded in the gelatine present, however, a very different appearance. The gelatine is liquefied by this fungus.

This species is incapable of fermenting either saccharose, dextrose, maltose, or lactose; neither does it invert saccharose. It was found in the slimy secretion on the roots of the elm, and shows considerable resemblance to the species *Mycoderma cerevisiae* and *Mycoderma vini*, but it is a true *Saccharomyces*.

The maximum temperature for spore-formation is 33° to 33½° C.; the minimum temperature 3° to 6° C., the optimum lying near 30° C. (17 to 18 hours). (Nielsen.)

It is impossible to prepare an asporogenous variety of *S. membranæfaciens* by cultivation at any temperature lying between the maximum for spore-formation and the maximum for budding. (E. C. Hansen.)

Koehler found this species in highly-polluted well-water. Pichi has described two species which very closely resemble *Sacch. membranæfaciens*.

In the writer's laboratory the species was detected in bright wines.

Pichi has described two species, one of which, *Pichia membranæfaciens* II., or *S. membranæfaciens* II., is found on the leaves of *Euonymus europæus*; the other, *Pichia membranæfaciens* III., or *S. membranæfaciens* III., was prepared in a pure state from a wine (Vin des Côtes). Seifert, again, has described three species, *Pichia californica* or *S. membranæfaciens*, var. *californicus*, from a Californian red wine; *Pichia taurica* or *S. membranæfaciens*, var. *tauricus*, from Crimean wine; and *Pichia Tamarindorum* or *S. membranæfaciens*, var. *Tamarindorum*, which was observed on tamarind pulp, and thence found its way into the wine-like drink prepared from it.

*Pichia farinosa* or *S. farinosus* was discovered by Lindner in Danzig Jopen beer (53° to 54° Balling), and by K. Saito in Soja sauce. The cells are slim, the older cells often angular. In the film abundant spore-formation takes place. The maximum temperature for the formation of the film is approximately 37° C. This species liquefies wort-gelatine when allowed to stand for some time. It feebly ferments dextrose and lävulose, but not *d*-mannose.

*Pichia Radaisii* or *S. Radaisii* is described by Lutz, and is found in "Tibi." It is an exciter of fermentation contained in the fig cactus (*Opuntia*), which is used in Mexico in the preparation of a feebly acid and alcoholic drink. The cells are a longish oval, the spores round, and usually four in each cell. At 22° to 23° C. spores form in twelve hours. The maximum temperature for spore-formation lies between 25° and 28° C.; the optimum temperature for film-formation 23° C. Development ceases at 37° to 38° C. Colonies on gelatine gradually acquire a red colour.

**Willia anomala or Saccharomyces anomalus Hansen**  
(Figs. 57 and 78).

This very curious species was found by Hansen in an impure brewery yeast from Bavaria. It gives a rapid and vigorous fermentation in wort, and even at the beginning of the fermentation develops a dull grey film. During fermentation the liquid acquires an ethereal, fruity odour (according to Seifert, ethyl acetate).

It brings about the decomposition of alcohol to form water and carbon dioxide, and finally decomposes the acetic ether. According to Nielsen, it only produces 0.9 per cent. by volume of alcohol in wort. It ferments dextrose, but neither maltose nor lactose, and secretes scarcely any invertase. Other observers have, however, found a distinct formation of invertase.

The cells grown in wort are small, oval, or sometimes sausage-shaped, and in their microscopic appearance they resemble species of *Torula*. When the development has gone on for some time many cells, both in the sediment and in the film, are found to contain spores.

An asporogenous variety has been developed by Hansen by cultivation at a temperature lying between the maximum for spore-formation and the maximum for budding. When *S. anomalous* is cultivated near the limiting temperature for budding, it grows only as sedimentary yeast. The temperature limits for budding in wort are 37° to 38° C. and 1° to 0.5° C.

According to Will, the walls of the young cells are coloured black with 1 per cent. of osmic acid. This, however, does not occur if the cells have previously been treated with alcohol. Many of the cells enclose large oil drops, which especially occur in cells derived from gypsum blocks that do not yield spores.

Spores are developed on various substrata, both liquid and solid, even when abundant nutriment is present.

The form of the spores is highly characteristic (Fig. 78); it resembles a hemisphere with a projecting rim round the base. On germination the spores swell and develop buds (see Fig. 57).

The maximum temperature for spore-formation is 32.5° to 34° C.; the minimum temperature 3° to 6° C.; the optimum is 30° C. (17 to 19 hours). (Nielsen.)



Fig. 78.—Spores of *Saccharomyces anomalous* (after Hansen).—Some spores are free, others inclosed in the mother-cells. On the right-hand side three spores are surrounded by the burst wall of the mother-cell.

After Hansen had drawn attention to this curious *Saccharomyces* species, this or similar species were observed by many workers. Thus, for instance, Meissner undertook a very comprehensive morphological and physiological investigation of three different species from Johannisberg must, from beer, and from samples of New Zealand soil. He described the form of the cells (in two cases round and in the third very elongated), the appearance of the films, the fermented liquors, and the giant colonies. The pale yellow must passes gradually into a dark brown, and the liquid gives an alkaline reaction. Both formation and decomposition of acid takes place. Reference may be made to Meissner's work on species of *Mycoderma vini* carried out at the same time.

Steuwer described four different kinds of *S. anomalous*, three of which produce acetic ether, whilst he states that one produces

both acetic ether and acetic acid. Lindner gave a description of a species found in Belgian beer, *S. anomalous*, var. *Belgicus* or *Willia belgica*. This ferments none of the known sugars, and produces no esters. Lindner also discovered a species in Mazun. Saito and Kozai discovered *S. anomalous* in Saké. Inui discovered it in Awamori, and believes that it imparts to this drink its peculiar aroma. Barker found *S. anomalous* by introducing ginger root into Mayer's sugar solution. Harrison has often found it in milk; and Holm discovered *S. anomalous* species in distillery mash, East Indian cane-sugar molasses, and in margarine.

In English high-fermentation beers which were "fretty," the author observed a species belonging to this group, which was multiplying very freely, so that all other yeast-cells had been suppressed. It appears distinctly as a disease-yeast causing turbidity in beer.

As previously mentioned, the spores of this fungus resemble those of *Endomyces decipiens*, and a relationship possibly exists between the two. As yet, however, no proof has been forthcoming in support of this assumption.

#### **Willia Saturnus or Saccharomyces Saturnus Klöcker**

is a species discovered in a sample of soil from the Himalayas. It forms a white film on liquids. The cells are chiefly oval or round. The temperature limits for budding in wort are 35° to 37° C. and 2° to 4°. The spores are lemon-shaped, with a vein running down the middle from end to end, and with a refractive granule in the centre. It ferments dextrose, laevulose, raffinose, and saccharose, but neither maltose nor lactose. It produces an ester during fermentation. Similar species were subsequently found in samples of soil from Italy and Denmark. Holm has detected it in a sample of soil from Japan.

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#### **Saccharomyces acidi laetici Grottenfelt.**

Grottenfelt has described under this name a species of *Saccharomyces* which, when added to sterilised milk, produces a pronounced curdling with formation of acid. On gelatine and

agar-agar it forms white porcelain-like colonies, and on potatoes it yields broad, moist patches of a whitish-grey colour, soon turning brown. In stab-cultures in gelatine, short bottle-shaped growths develop from the point of inoculation inwards. The cells are elliptical, 2.0 to 4.35  $\mu$  in length, and 1.5 to 2.9  $\mu$  in breadth.

When a solution of milk-sugar is inoculated in the presence of calcium carbonate, and the product distilled, alcohol can be detected. In a neutral 3 per cent. solution of milk-sugar, *Saccharomyces acidi lactic* yielded 0.108 per cent. of acid in eight days.

***Saccharomyces fragilis* Jörgensen (Figs. 79 and 80).**

While the budding and lactose-fermenting fungi found in kefir, and described by others, do not form spores, a genuine *Saccharomyces* has been discovered in the author's laboratory,

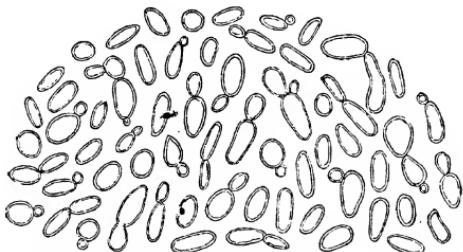


Fig. 79.—*Saccharomyces fragilis*.—Young growth in lactose yeast-water (drawn by Holm from nature).

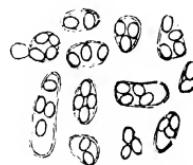


Fig. 80.—*Saccharomyces fragilis*.—Spore-formation (drawn by Holm from nature).

which has been called *Saccharomyces fragilis*, on account of the feeble power of resistance of the cell-wall.

The growth consists of relatively small, oval, and longish cells (Fig. 79), with curious and feeble refraction. At room temperature, this species behaves as a low-fermentation yeast. In cultures on gypsum blocks distinct spore-formation begins in 20 hours at 25° C., and in 40 hours quite a number of free spores may be observed (Fig. 80); at 15° C. the spore-formation takes place in about 40 hours. The bean or kidney shape of the spores is characteristic. Spores are also formed in growths in fermenting liquids and on gelatines,

and in every case are soon set free. After long standing, the growth forms a thin film, the cell-forms of which deviate comparatively little from those of the sedimentary yeast. In plate-cultures the surface colonies formed in the course of two or three days at room-temperature are film-like, and fuse together, while the embedded colonies exhibit thickly-haired, mycelial borders.

In lactose yeast water (10 per cent.), at room temperature, this species yielded about 1 per cent. by weight of alcohol in the course of eight days. In two months as much as 4 per cent. by weight of alcohol was produced. At the same time the formation of acid began. In hopped wort (about 11 per cent. Ball.) it yielded at the room temperature about 1 per cent. by weight of alcohol in ten days.

The optimum temperature for development lies at about 30° C.

According to Bau, this species ferments milk-sugar completely, but not melibiose.

Holm found similar species with kidney-shaped spores in milk and in agave juice.

In "Sauer" (sour whey, used for the preparation of rennet in the manufacture of Emmenthaler cheese) Freudenberg and Orla Jensen found a *Saccharomyces* which produced a pleasant alcoholic odour in cream and formed spores in 23 hours at 25° C. Orla Jensen isolated two species of *Saccharomyces* from Swiss butter. They ferment maltose and lactose. One forms spores in 24 hours at 25° C., and in three days at 30° C.; the other only forms spores in six days at 30°. In both cases the spore-formation is a scanty one. P. Mazé isolated a species directly fermenting lactose from cheese (Port du Salut), which forms spores in 24 hours at 26° C.

#### ***Zygosaccharomyces Barkeri Saccardo et Sydow***

is a yeast species isolated by Barker from ginger. It can be recognised by the fusion of two cells preceding spore-formation. The development can be observed under the microscope in hanging drops of sterile water. Many of the cells will be found to produce beak-like protuberances in twelve hours at 25° C.

(Fig. 81). The prolongations of two neighbouring cells grow towards each other until they come in contact. At the place of contact the projections fuse together, and the protoplasm of the two cells flows together. At a later stage the protoplasm separates in each cell, and two rounded corpuscles appear which become spores. According to Barker, staining shows that this process is accompanied by a fusion of the cell nuclei, and Barker, therefore, denominated it a sexual process. According to Guilliermond, the conjugation of the *Zygosaccharomycetes* is exactly identical with that of *Schizosacch. Pombe* and *Schizosacch. mellacei*. The nuclei of the two cells fuse together always in one of the cells, and never in the conjugating passage. It may be assumed that in the formation of asci in conformity with the *Ascomycetes* an isogamous conjunction takes place.

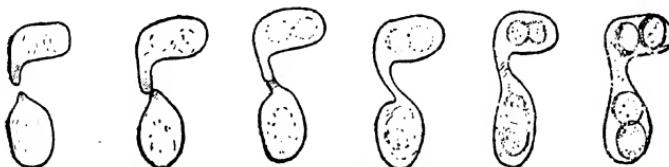


Fig. 81.—*Zygosaccharomyces Barkeri*.

The spores swell up, and cause the walls of the mycelium to burst, and then the spores develop in the usual way by means of budding. On gypsum blocks and on gelatine the development proceeds in a similar way to that already described. The spores are chiefly round, seldom oval, and rather strongly refractive. They form readily and rapidly at 25° to 23° C. The maximum temperature appears to be 37° to 38° C., and the minimum 13° C. Spore-formation may take place without fusion of the cells, as sometimes occurs in the case of *Schizosaccharomycetes*. The cells are oval; it forms no film, but a yeast ring develops in ten to fourteen days at 23° C. The cells in the latter are oval, and occasionally elongated. Spore-forming cells also occur in the ring. Colonies formed on the surface of the gelatine have a smooth edge, whilst the colonies immersed in the gelatine have a fringed edge.

Dextrose, laevulose, and saccharose are fermented, but neither maltose, lactose, nor dextrin.

*Zygosaccharomyces* appear to occur fairly frequently in nature. Since Barker drew attention to this peculiar genus of *Saccharomyces*, similar species have been found by other workers. Thus, for instance, Holm has found them on figs and pears, in dunder, and in Barbados sugar.

***Saccharomyces Ludwigii* or *Saccharomyces Ludwigii*  
Hansen. (Figs. 55, 56, and 82.)**

This remarkable species, which was discovered by Ludwig in the viscous secretion of the living oak, is the only one of the known *Saccharomyces* which can be recognised solely by means of a microscopic examination. The following description is taken from Hansen's investigations. The cells are very variable in size, elliptical, bottle-shaped, sausage- or frequently lemon-shaped. Partition walls may occur in all the complex cell-combinations. The vegetative growths in wort-gelatine are round like those of the majority of the *Saccharomyces*, and are either pale grey, or faintly yellow. In wort, it yields only 1·2 per cent. by volume of alcohol, even after a long-continued fermentation ; and this accords with the fact that maltose is not fermented by this species. In dextrose solutions, on the other hand, it yields up to 10 per cent. by volume of alcohol. It inverts saccharose, but does not ferment solutions of lactose and dextrin, neither does it saccharify starch paste. It readily develops spores in aqueous solutions of saccharose, in wort-gelatine, in yeast-water, and in wort ; in the latter case, even when no film has formed.

It is characteristic of this species that a fusion of germinated spores often occurs, especially in the case of young spores, and these new formations develop germ-filaments (proto-mycelium), from which new yeast cells are gradually marked off by sharp transverse septa (Figs. 55, 56). At the ends of these cells, buds develop, and these again are marked off by transverse septa.

According to Hansen, a stable asporogenous variety cannot be prepared by cultivation at temperatures lying between the maximum for spore-formation and the maximum for budding. On the other hand, Hansen found that a number of cells lost

their power of forming spores when they remained for some time on one and the same substratum. Other cells largely lose this power, and the remainder do not appear to be influenced. By cultivation in favourable liquids the first set of cells again yield spores. Beijerinck found that the colonies

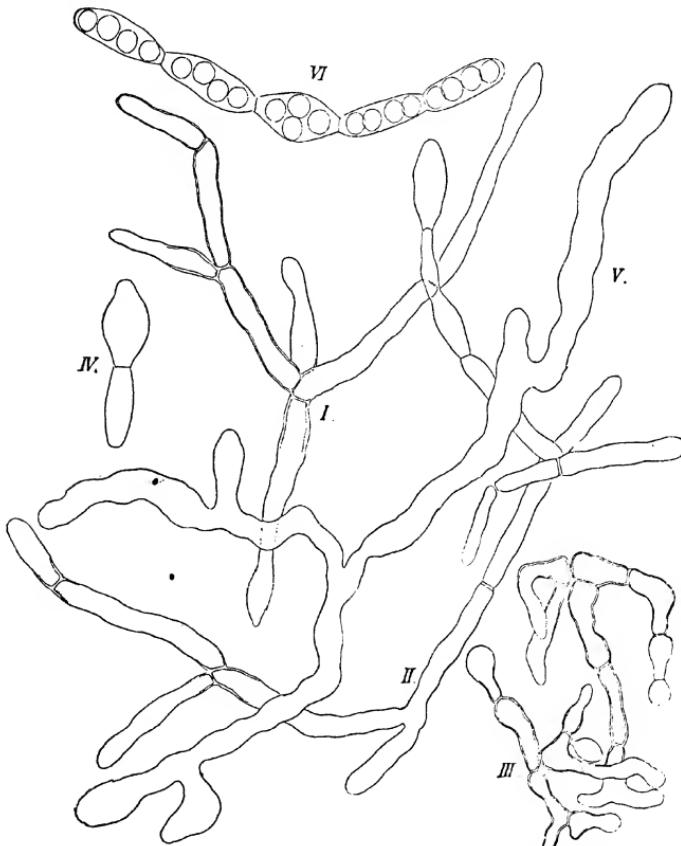


Fig. 82.—*Saccharomyces ludwigii*.—Old film and mycelium (after Hansen).

formed from asporogenous cells do not liquefy gelatine, which is contrary to what occurs with the sporogenous colonies.

Guilliermond, who also examined the germination of spores, records amongst other facts that spores which are not derived

from the same ascus may fuse together, and further that the spores which grow together by fusion may then develop to asci with spores without previously forming new cells (compare Hansen's experiments with wine yeast, *Johannisberg II.*). He finally showed that by the fusion and germination of spores a combination of their cell nuclei and a subsequent division takes place as soon as the promycelium has reached a certain size. Guilliermond described this process as conjunction with isogamy.

Whilst in the case of *Schizosaccharomyces* and *Zygosaccharomyces* a conjunction takes place between the vegetative yeasts converting these into an ascus, in this case conjunction takes place between the spores and even before they germinate.

Will discovered that the spores may sometimes be more or less distinctly stained to a bluish-green with iodine in potassium iodide (a similar colour to that given by *Bact. Pasteur. Hansen*).

In old cultures (Fig. 82) there is a strong tendency to form mycelia, but it is only exceptionally that portions are found where the links are closely bound together, and only display slight contractions. Such portions are provided with distinct and straight septa. Every cell of such a colony can form spores as well as buds. Amongst them odd shapes and very large, strongly branched cells are found. It is characteristic of this species that the cells die off within two years in aqueous sugar solutions, whilst most of the known Saccharomyces live much longer in this liquid. The temperature limits for budding in wort are 37° to 38° C. and 3° to 1° C. The maximum temperature for spore-formation is 32° to 32.5° C., the minimum 3° to 6° C., the optimum 30° to 31° C. (18 to 20 hours). (Nielsen.)

Holm has repeatedly found *S. Ludwigi* in fermenting apple and gooseberry juice, as well as in English and American cider. Behrens found a Saccharomyces on hops in which the germinal tubes of the spores fuse together as is the case with *S. Ludwigi*. The cells are large, round, and oval, the spores round and refractive. At 18° to 20° C. spores appear in 22 hours. No film-formation takes place. It ferments dextrose, lævulose, and maltose, but does not invert saccharose nor ferment lactose and galactose.

A series of recently discovered species closely allied to *S. Ludwigii*—the *Schizosaccharomyces*—are distinguished by the total disappearance of budding, and the fact that the propagation of the cells takes place by division.

**Schizosaccharomyces or Saccharomyces comesii Cavara**

was described in 1893. It lives as a parasite or saprophyte on the sheaths or pedicles of millet, and, according to Cavara, forms a mycelium consisting of cylindrical hyphae with partition walls; this mycelium produces cylindrical or longish ellipsoidal conidia, 7 to 8  $\mu$  long and 2 to 3  $\mu$  broad, isolated or linked together. In sugar solutions it produces a growth of yeast, and when the nutritive solution is exhausted, spores appear within the cells. These spores are globular, two to four in each cell. Two or more fuse together in the mycelium, through the membrane of which the germinal threads appear. This species resembles *S. Ludwigii* in that it has no typical budding fungus, but differs especially in that it occurs as a typical mould on its host.

*Schizosaccharomyces (Sacch.) octosporus* was discovered by Beijerinck on dried currants, and has been more recently examined in the author's laboratory by Schiönnning in growths on raisins. The propagation takes place in the following manner (Fig. 83). About the middle of the cell a partition-wall appears; after this has split up, the two new cells assume a round shape, and revolve round a point of the septum, where connection is still maintained, so that at last they lie almost parallel. Finally, they separate entirely from each other, having taken an ellipsoidal or oval shape; they then extend in length, and the division begins afresh. But it may also occur that two cells, still connected throughout the full extent of the partition-wall, increase in length and form fresh septa, so that the original mother-cell appears divided into four or more cells. The cells are 4.5 to 6  $\mu$  broad and 7 to 13  $\mu$  long. Even at the beginning of the fermentation in wort at 25° C. the cells form endospores; but the spore-formation is very feeble both under ordinary fermentative conditions in wort and also during cultivation on moist gypsum blocks. Seiter found, that the spores are formed on gypsum blocks in 6-7 hours

at 25° C. This development is much more vigorous on the surface of nutrient gelatines, such as wort-gelatine (Fig. 85), where it forms round, waxy, and raised colonies with a depression in the centre. The cells grow shorter and more rounded after developing for some days at the temperature of the room, and the ascus-formation, according to Schiöning's observations in the Carlsberg laboratory, now takes place as follows (Fig. 84) :—

The rounded cell lengthens; a partition - wall appears, which splits off, after which the two new cells merely touch or connect at one point. They then, again, coalesce (compare the fusions observed by Hansen in *S. Ludwigii*), and at last

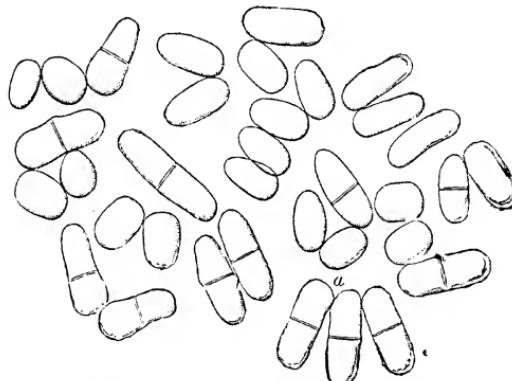


Fig. 83.—*Saccharomyces octosporus*.—Young growth after cultivation for twenty-four hours in beer-wort, at 25° C. (after Schiöning).

form a lengthened, ellipsoidal, hour-glass shaped or irregular cell, which gradually increases in bulk (frequently 14 to 20  $\mu$  long). In these cells eight spores form, as a rule, but frequently only four, and less frequently from two to seven. By degrees the wall of the mother-cell dissolves, and the spores now lie embedded in slime, which subsequently disappears. The spores are often oval, and, according to Lindner, their membrane is coloured blue by a solution of iodine in potassium iodide. According to Guilliermond, the ascus - formation may also proceed in this way. Two cells which have not been derived from one and the same cell fuse together, or again an ascus may be formed without the fusion of two cells.

Both on the vegetative cells and on the asci of the spores fine lines may sometimes be observed, which form the limit between the older, thicker parts of the cell-wall and the newly-formed, thinner parts. The latter appear after the partition-walls, which now form the terminal walls of the new cells, have divided, or after fusion, through the ensuing growth of the ascus.

On wort no film has been observed; only a slender yeast-ring.

Wort-gelatine is rapidly liquefied by *S. octosporus*. Beijerinck found that in the case of the asporogenous cells the for-

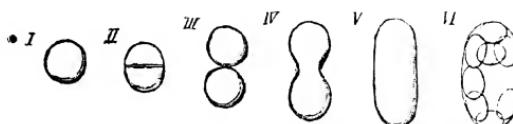


Fig. 84.—*Saccharomyces octosporus*.—Development of the ascus (after Schiönnig).

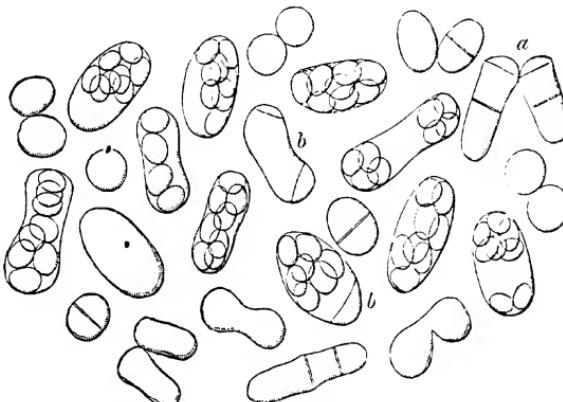


Fig. 85.—*Saccharomyces octosporus*.—Young growth in wort-gelatine (after Schiönnig).

mation of trypsin is gradually reduced, while the formation of acid is greater than in the sporogenous cells.

This species ferments maltose, levulose, and dextrose, but it does not invert saccharose. According to Fischer, an aqueous extract of the dried pulverised growth decomposes maltose, but does not exert any influence upon saccharose. He states that it contains raffinase, and is, therefore, capable of fermenting raffinose.

*Schizosaccharomyces (Sacch.) Pombe* was discovered by Saare and Zeidler in millet beer from Africa, and more exactly described by Lindner. It is closely allied to the previous species; its propagation also takes place by formation of partition-walls and by fission; frequently the two new cells remain connected for some time at a single point, upon which they rotate until they form an acute angle to each other. The cells resemble the conidia of *Oidium*; but the shape of many of them is suggestive of the manner in which they were derived, one end being rounded, whilst the other is surrounded by a well-defined ring-wall, enclosing the newly-formed piece of globular membrane. In the cells one to four spores may occur, which grow in the same way as those of *S. Ludwigii*—viz., by the formation of a germinative thread—no fusion of the promycelium of the spores has been observed.

Guilliermond has closely examined the course of spore-formation, and finds that the ascus-formation often follows a fusion of two cells which may be sister cells. In this way the dumb-bell shaped cells often met with in spore-cultures are formed. He also observed a fusion of three cells. Spores also form in the sedimentary yeast. By germination they swell up and form a germinal tube, which afterwards divides into two cells by means of a septum.

The growth forms no film on wort. On wort-gelatine it forms a compact finely-furrowed growth.

At its optimum temperature, 30° to 35° C., this species shows high-fermentation phenomena. It is distinguished by the considerable amount of acid formed during the fermentation, and possesses a certain power of resistance in competition with bacteria. In beer-wort it gives a rather vigorous fermentation; it also produces fermentation in dextrose, maltose, and cane-sugar solutions. It does not ferment *d*-mannose, but does ferment dextrin.

According to Rothenbach's experiments, it ferments about half the total amount of diastase-dextrin prepared according to Lintner's directions, leaving achroo-dextrin, which, on addition of alcohol, slowly separates out in sphæro-crystals.

As this species is capable of forming very considerable amounts of alcohol, it might be supposed to be of industrial

value. Experiments made in this direction, however, have hitherto proved unsuccessful.

Lepeschkin often found mycelial formations in young cultures of *S. Pombe*. This formation remained through countless generations. He believes that the mycelium is not to be regarded as a normal form of development, but only occurs through the reconstruction of cells. The requisite conditions are unknown.

In the rum-fermentation of molasses in the West Indian Islands, two different yeast types occur. In a few districts the common ellipsoidal form predominates; in other districts a mould-like *Saccharomyces*. In arrack-fermentation of molasses in Java Vordermann and Eykmann constantly found a fungus which separates new cells through formation of partition-walls; no spore-formation was observed, and, according to Eykmann, the fungus recalls *Hyphomycetes* in its growth forms. A *Saccharomyces* of similar appearance was discovered by P. Greg while working in the writer's laboratory in cane-sugar molasses as used in rum-fermentation in Jamaica. This is designated:—

**Schizosaccharomyces or Saccharomyces mellacei**  
**Jörgensen. (Figs. 86 and 87.)**

In cylindrical vessels at 25° C. this species ferments beer-wort with top-fermentation phenomena, forming a caseous, loose deposit. During fermentation it develops a pleasant aroma. In wort of 10.5 per cent. Ball. it produces about 2½ per cent. by weight of alcohol.

The different forms assumed by the species, recall *Saccharomyces octosporus*, *Sacch. Pombe*, etc. In old cultures very curious cell-forms (Figs. 86, 87) occur, which also develop during fermentation. In wort-cultures five months old no film had developed; only a yeast ring was observed. The liquor is not decolourised by old cultures.

The spores (Fig. 87) are oval. They occur in all cell-forms, generally four to a cell; they refract light strongly, and, according to Holm, they are coloured blue by iodine.

Guilliermond found that an ascus is produced with this

species by the fusion of two cells which are often sister cells, but ascus-formation may take place without previous fusion.

In plate and streak-cultures the growths, both on and

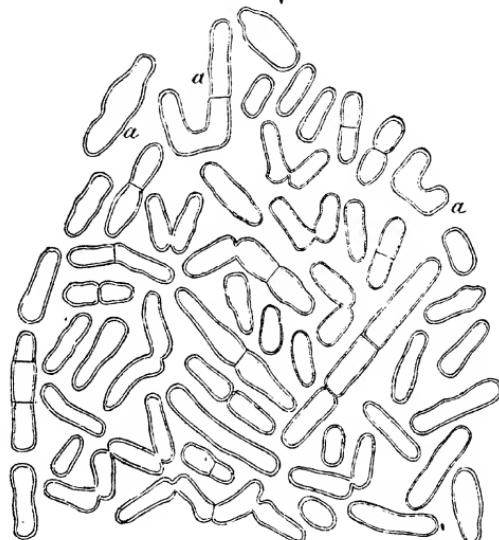


Fig. 86.—*Saccharomyces mellacei*.—Young culture in beer-wort; *a*, cells after eight days' cultivation (drawn by Holm from nature).

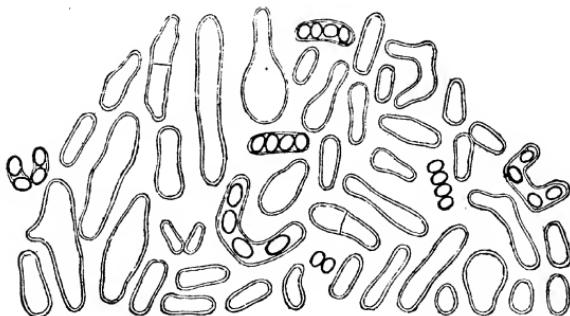


Fig. 87.—*Saccharomyces mellacei*.—Growth from the yeast ring in beer-wort (drawn by Holm from nature).

below the surface have a sharp-cut edge; the cell-forms are similar to those in liquids; cells shaped like the conidia of *Oidium lactis* frequently occur.

It ferments dextrose, maltose, and saccharose, as well as *d*-mannose and dextrin. According to Lepeschkin, *S. mellacei* may also form a mycelium (see *S. Pombe*).

According to investigations made by P. Greg in the author's laboratory, divergencies of a marked and permanent character distinguish the species belonging to this type. Thus some yield malodorous products in the fermenting liquor, some very fine products, whilst others differ greatly in the length of time required to complete the fermentation in one and the same sterilised molasses and dunder under identical conditions. The amount of alcohol produced by these types varied from 6.6 to 7.6 per cent. by volume. The rate of multiplying also differed widely in these species. Further details relating to comparative results in practice are given in papers by Greg as well as by Hart, who has carried out rum-fermentation with ellipsoidal species.

In the author's laboratory *Schizosaccharomyces* have been found by Holm in Grecian wine must, on cocoa beans, and on Mohwa flowers from Cawnpore.

**Saccharomyces guttulatus or Saccharomyces guttulatus**  
(Robin) Wilhelmi

was found by Remack in 1845 in the contents of the stomach and intestines of a rabbit, and subsequently described by Robin under the name *Cryptococcus guttulatus*.

Both classified it amongst the yeast fungi. In 1896 Buscalioni gave a comprehensive morphological description of the organism, which he named *S. guttulatus*. We are indebted to Wilhelmi for the following description (Schiönning has associated this form with one described by him as *Saccharomyces capsularis*). The cells are elliptical and longish-oval with blunt ends. The length varies from 6 to 16  $\mu$ , width 2 to 4  $\mu$ . They contain abundant quantities of glycogen. With poor nourishment, from two to four large and strongly-refracted vacuoles are observed. Budding is linear or spiral. Under favourable conditions of nutriment the buds will be



Fig. 88.  
*Saccharomyces*  
*guttulatus*.

detached at an early stage of development; if the conditions are unfavourable, bushy colonies are formed. In the mother-cell from two to four elongated oval spores are formed, provided with two membranes (exosporium and endosporium) (Fig. 88).

On germinating, the exosporium bursts near one of the poles, or at the side, always with an irregular edge, and crumples up to form a small indistinct residue, which usually clings to one end of the endosporium. Germination and growth of the spores takes place in presence of from 1.25 to 5 per cent. of hydrochloric acid and 10 per cent. of sugar, at a temperature of 37° C., whilst spores are formed at a temperature of 14° C.

#### *Saccharomyopsis capsularis* Schiönnig

was discovered in an analysis of soil. The sample was taken from the neighbourhood of the St. Gotthard Pass. The youngest sedimentary growths in beer-wort consist of variously shaped cells, especially *Pustorianus* forms, often having stumpy ends. Within two days small islands of a film appear on the surface of the liquid, consisting of typical-branched mycelium with septa, partly breaking into bud and partly separating into round or *Oidium*-like links. At a later stage a few mycelium forms are found in the sedimentary yeast. The covering becomes thick and uneven with a dry, white, and slightly hairy appearance. In these surface cultures a few spores occur later, especially in the round cells or *Oidium*-like cells formed at the end of the threads, but occasionally in the mycelium threads themselves. These spore-forming cells contain a specially bright refractive protoplasm; four spores almost always form in each ascus. They are coloured bright rosy red with concentrated sulphuric acid. The spores have a flattened spherical shape surrounded with a fine transverse line. During germination it can be recognised that the spores are provided with a double wall (exosporium and endosporium). The outer opens up into two unequal valves and divides along the transverse line. The two valves are fastened together at one point, and lie like a pair of mussel shells round the swollen cells, which soon begin to bud. It is the exosporium that gives a red coloration with sulphuric acid and other mineral acids. It probably contains cork.

On the surface of wort-gelatine agar greyish-white colonies form with a slightly hairy appearance. They gradually change to a chocolate-brown colour. On gypsum blocks spore-formation is not so abundant as on solid yeast water substrata.

It ferments maltose, dextrose, levulose, and *d*-galactose, but not *l*-arabinose, raffinose, lactose, or saccharose: saccharose is also not inverted. In ordinary beer-wort (about 13.3 per cent. Balling) it can produce 7 per cent. by volume of alcohol.

Optimum temperature for vegetative growth 25° to 28° C.; maximum temperature 38.5° C.; minimum under 0.5° C. Optimum temperature for spore-formation 25° to 28° C.; maximum 34.5° to 35° C.; minimum between 5° and 8° C.\*

## II. BUDDING FUNGI WITHOUT SPORE-FORMATION.

### Torula.

These yeast-like forms were first characterised by Hansen. They are widely distributed, and, therefore, not infrequently occur in physiological analyses connected with fermentation. They occur in both spherical and more or less elongated forms, and are distinguished from the genus *Saccharomyces*, as first pointed out by Hansen, by their inability to form endogenous spores. In most cases they multiply only by budding, in some few cases also by the formation of mycelium.

According to the author's researches, certain *Torula* forms may act as disease-yeast, for they multiply freely and give rise to a kind of turbidity in weakly fermented, high-fermentation beers, when these are bottled; the character of this turbidity, however, is somewhat different from that caused in low-fermentation beer by the wild *Saccharomycetes*.

In sugar-works, the writer finds *Torula* forms occurring extensively, frequently in large quantities, even in the finished

\* Certain dubious species of *Saccharomycetes* must be mentioned, one isolated by Metschnikoff, *Monospora cuspidata*, occurring as a parasite in *Daphnia*, which has long-cells and contains needle-shaped spores, and another, *Nematospora Coryli*, isolated by Peglion from hazel nuts, containing eight spores to a cell, in two bundles, each of four spores. The spores carry a long flagellum at each end, which disappears before the germination of the spore takes place.

product. Among the species examined many possess an inverting enzyme. It is not improbable that these growths assist in the progressive formation of invert-sugar which frequently takes place during the storage of cane-sugar.

Hansen has observed many different species, and has described the following in detail :—

The *first* occurs in wort, the cells being either single or in small clusters. Some cells have a large vacuole in the middle, and this sometimes contains a small strongly-refractive particle. The size of the cells varies considerably (1.5 to 4.5  $\mu$ ). This species does not secrete invertase, and causes a scarcely perceptible alcoholic fermentation in beer-wort.

Under the same conditions the *second species* possesses larger cells than the first (3 to 8  $\mu$ ) ; they resemble the foregoing, except that the contents of the cells grown in wort are often very granular.

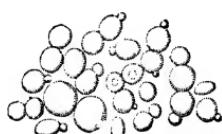


Fig. 89.—*Torula* (after Hansen).—Sedimentary forms after one day's cultivation in beer-wort at 25° C.

The *third species* which, microscopically, resembles the last, produces under the same conditions as much as 0.88 per cent. by volume of alcohol; it gives a distinct head with evolution of carbon dioxide, but it cannot invert cane-sugar.

The *fourth species* (2 to 6  $\mu$ ) inverts cane-sugar and produces slightly more than 1 per cent. by volume of alcohol in wort with considerable frothing ; it does not, however, ferment maltose.

The *fifth species*, which in the form and size of its cells resembles the first, develops a uniform, dull grey film on wort and yeast-water at the ordinary room temperature, likewise on lager beer, and even on liquids containing as much as 10 per cent. of alcohol. It inverts cane-sugar, and forms a slight film on the solution. It does not, however, excite any appreciable alcoholic fermentation.

A *sixth species* (Fig. 89), which forms spherical and oval cells, gives a distinct fermentation in beer-wort, yielding as much as 1.3 per cent. by volume of alcohol. It does not ferment maltose solutions. It inverts cane-sugar, and in 10 per cent. and 15 per cent. solutions of this sugar in yeast-

water, it yields respectively 5.1 and 6.2 per cent. by volume of alcohol, after fourteen days at 25° C.; the latter culture gave 7 per cent. by volume of alcohol in two months. Dextrose solutions of the same concentration and under similar conditions gave 6.6 and 8.5 per cent. of alcohol by volume.

The *seventh species* (Figs. 90 and 91) was found in the soil under vines. The sedimentary cells are most frequently oval and in part larger than those of the last species. Certain



Fig. 90.—*Torula* (after Hansen).—Sedimentary forms after one day's growth in beer-wort at 25° C.



Fig. 91.—*Torula* (after Hansen).—Same species as Fig. 90. Film-formation on a wort-culture ten months old.

cells of the films are very irregular in shape. This *Torula* produces only 1 per cent. by volume of alcohol in wort, does not ferment maltose, and neither ferments nor inverts cane-sugar. In 10 per cent. and 15 per cent. solutions of dextrose in yeast-water it gave 4.6 and 4.5 per cent. by volume of alcohol in 15 days at 25° C., and 4.8 and 4.7 per cent. in 28 days. In two other flasks 4.8 and 5.3 per cent. of alcohol were produced after long standing. Hansen assumes that this

species takes part in vinous fermentation, and considers it probable that species such as the sixth and seventh, which produce a vigorous fermentation in dextrose solutions, take part in the fermentation of grape and other fruit juices. On the other hand, they have probably little importance in breweries and distilleries, since they are unable to ferment maltose.

Another species of *Torula* (*Torula Novæ Carlsbergiæ*), the cells of which exhibit very different forms, has been described by Grönlund. It imparts a disagreeable bitter taste to wort. According to Schjerning's investigations it inverts cane-sugar, and induces alcoholic fermentation in solutions of cane-sugar, dextrose, and maltose. In ordinary brewery-wort it can produce about 4.7 per cent. by volume of alcohol.

The following species of *Torula*, together with the two rose-coloured budding fungi described under the name of *Torula b* and *c*, were prepared in a pure state in the author's laboratory, and are used in the course of instruction given in his laboratory.

J. C. Holm was the first to give an exact description of these three species.

*Torula a* = *Torula Holmii* n.sp.

The culture of the young sedimentary growth, consists of small oval cells (Fig. 92). Here and there single larger cells occur both oval and round. The length of the cells varies from 3.5 to 5.5  $\mu$ , the breadth from 1.4 to 2.1  $\mu$ . It gives a feeble fermentation in wort, yielding about 0.32 per cent. by weight of alcohol. It inverts saccharose and raffinose, and ferments the inverted sugars. It ferments dextrose, but not maltose, lactose, and dextrin (d. puriss. Kahlbaum). A film-formation takes place in wort in three to five days at 25° C. The cells of the film are round and oval in wort, whereas in dextrose-yeast water they assume *Pastorianus* or irregular forms. The surface colonies on wort-gelatine (10 per cent.) are round, white, lustrous, and slightly raised. The edge of the colonies is smooth.

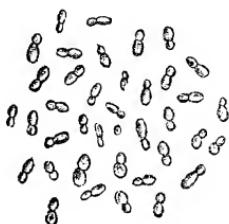


Fig. 92.—*Torula a.* Young culture (Brask).  $\times 560$ .

Schiönning submitted the *Torula* found by Claussen in English beer to a close examination, during which he isolated from different English beers some other species belonging to the same group. He found that they fall naturally into two distinct groups. Schiönning selected a typical representative of each, described temporarily as *Torula (A)* and *Torula (B)*, and gave a comprehensive description of both types.

Both develop slowly in ordinary lager-beer wort. They give a typical low fermentation, which, in the case of *Torula (B)*, lasts for six months at 20° C., whereas (A) ferments somewhat more rapidly.

*Torula (A)*.—The cells are elliptical, but at the same time sausage-shaped and even mycelial forms occur together with queer erratic shapes. The size is somewhat variable. Giant cells with strongly refractive protoplasm and thick walls are also found. The protoplasm of the ordinary cells is feebly refractive, with indistinct vacuoles frequently containing a motile body.

*Torula (B)*.—The cells look somewhat like *Torula (A)*, but are on the whole rather slimmer and more uniform, for the most part sausage-shaped, but long mycelial cells also occur. In older cultures a loose layer of mycelium consisting of threads covers the true sedimentary yeast layer.

In sterilised beer, which in the case of *Torula (B)* must be mixed with a little saccharose or glucose, a slow propagation takes place at 25° C. The beer at first thickens, and afterwards gradually clarifies with the simultaneous formation of a somewhat coherent sediment. Its cells are, on the whole, larger and more uniform, and the protoplasm is more refractive than in the corresponding wort-cultures. If the development has taken place in closed vessels the beer gives evidence of a high content of carbon dioxide when it is poured out, and gives a fine head. By storage in flasks with access of air both species form a feeble yeast ring and a film (like *Saccharomyces*) ; the cells are elongated.

*Torula (A)* may sometimes occur with a fine, dry, and greyish film like *Mycodarma*. The cells are then regular and elliptical. Nothing is known as yet regarding the conditions for such formation. It is probably derived from individual differences in the cells.

The following notes may be given regarding their physiological properties :—

The temperature limits for propagation are—*Torula* (A) 40° to 40.5° C. and 5° to 7° C.; *Torula* (B) 39.5° to 34° C. Optimum temperature: 30° to 35° C. for both groups. They have practically the same limits of temperature for existence. It is worthy of note in this connection that the cells die in fifteen to eighteen months in wort, but if a little calcium carbonate is added life may be preserved for a long time, probably in consequence of the neutralisation of acid formed by the cells. In bottled beer they can live for a long time.

With respect to their reaction on sugars, it has been proved that saccharose is easily fermented by (A), and less rapidly by (B). Glucose and levulose are easily fermented by both; maltose more readily by (A) than by (B). Neither ferments dextrin. Saccharose-yeast-water is fermented, but without giving Fehling's reaction, for although inversion takes place, the inverted sugar is immediately fermented. Lactose is fermented only by *Torula* (B). It is easy to distinguish the two groups through their physiological behaviour.

They are not very sensitive to the alcohol and acid formed during fermentation, so that at the end of the primary fermentation they can hold their own in competition with *Saccharomyces*, and may, therefore, ferment the sugar residues with which the *Saccharomyces* are incapable of reacting.

When *Torula* (A) is added to fully-fermented beer (Danish export beer and Danish stout) or to wort, it forms acid along with alcohol, which to some extent combines with the alcohol and produces characteristic aromatic and flavouring ethereal substances. The acid reaction of the liquid is also increased. *Torula* (B), on the other hand, cannot develop further in these beers, but by the addition of sugar a fresh fermentation sets in, with peculiar aroma and flavour. This *Torula* is, therefore, unable to ferment the sugar residues if the beer has already been well fermented.

In addition to these two species, Schiönnning isolated from English beers about 150 forms belonging to this group, possibly only varieties or races of the typical species described. Most of them are closely related to the *Torula* (A) group. Certain

of these forms must be regarded as disease organisms, for they immediately form a dry film, which afterwards, on shaking, renders the beer turbid. In Danish, Swedish, and American beers these species can also be detected. The conditions, however, are extremely unfavourable for their development; this is especially the case with the low temperatures used in the preparation of lager beer. According to Schiönnig, if pure cultures of these forms are introduced into Continental beers, they appear as true disease forms, imparting an unpleasant taste and smell to the beer.

We are indebted to Will for one of the most complete investigations of *Torulas*. His very comprehensive experiments concern themselves, on the one hand, with a morphological description of the species during their cultivation on different liquid and solid substrata, and with observations on the influence of temperature (temperature limits for growth of cells and influence on their shape). On the other hand, he observed the behaviour of the species in competition with different wild culture yeasts; their reactions with the sugars; their power of forming glycogen in different liquids; their production of acid, and their power of producing sulphur-ated hydrogen.

He mentions fourteen different species derived partly from air and water analyses, and partly from the laboratory. Some are found in beer and some on grapes. They all develop freely in hopped wort, even at low temperatures such as may be used in the primary fermentation. They develop distinct differences in flavour and aroma, the latter often aromatic, but in some cases unpleasant. A slight decolorisation of the wort takes place. The acidity is diminished in the majority of cases, but increases with certain species. The majority are suppressed when brought into competition with different low-fermentation culture yeasts, even when large amounts are used for inoculating, and this takes place in the primary fermentation. Nevertheless all the species do not behave alike in this respect, and again the various races of culture yeasts are unequal in their power of suppressing the *Torulas*. The *Torulas* have no influence on the taste and odour of lager beer, and they do not bring

about beer turbidity or reduce the stability of beer, even when vigorous cultures are added to the beer as prepared for consumption, if it is kept in well-filled and stoppered bottles. If *Torulas* are added in minute quantity at the beginning of storage, they display no development during storage, and die off towards the close. It is clear that their development is not hindered by the quantity of alcohol produced, from the fact that they grow in yeast-free beer with access of air. It is rather the lack of oxygen and the quantity of carbon dioxide present that are accountable for their suppression.

In respect to the reaction of the *Torulas* on the sugars, Will proved that the small-scale fermentation method of P. Lindner is not applicable. The question to be solved is whether an organism entirely lacks the power of fermentation, or whether a given sugar is unfermentable. The experiments were, therefore, carried out with larger quantities of sugar in Erlenmeyer flasks. The following sugars were tested:—Dextrose, laevulose, maltose, saccharose, galactose, and lactose, dissolved in neutral yeast water. In all these solutions the species investigated grew equally well, and proved that they were capable of assimilating those carbohydrates which they are unable to split up into alcohol and carbon dioxide. All the species examined fermented dextrose, laevulose, saccharose, and galactose, and some of them could also ferment maltose, but in other cases only small quantities of alcohol were produced. Lactose was fermented to an extremely small extent by two of the species.

Van Hest found a small (4 to 5  $\mu$ ) oval or almost round *Torula* in top-fermentation Dutch beer, partly in the beer returned, partly in beer in the lager casks, and again in the fresh beer at the end of the primary fermentation. All these were opalescent or turbid, and possessed a peculiar fruity flavour. He termed the *Torula*, *Sacch. pinophtorus melodus*, and found that under varying conditions its shape varies greatly, and that a mycelium is found in the film. It is the cause of the diseases of beer just described. In wort it brings about a fairly strong fermentation. Another *Torula* (*S. pinophtorus enervans*) is often found in the same beer. It is

even smaller (2 to 6  $\mu$ ) and round, produces less alcohol, and gives no aroma. The taste and odour of the beer is not spoilt to the same extent as in the previous case.

Meissner found certain *Torulas* in old bottled wines and in viscous wines, which he termed "slime yeasts." He described eleven forms, some round, some oval; others large like *Pastorianus*. They do not ferment, but turn must, wine, and other liquids slimy. The addition of ammonia greatly increases their growth, and brings about the viscosity at an earlier stage. Larger quantities of alcohol hinder growth; this is also the case with the addition of sulphurous acid in the form of potassium bisulphite, even with the small quantity of .05 per cent. A similar effect is produced by the addition of .06 per cent. of tannic acid. Feebly-fermenting yeasts are at first suppressed by the slime yeasts, but at a later stage when the percentage of carbon dioxide increases these are themselves suppressed. For wine fermentation it is, therefore, of importance to use strongly fermenting yeasts. It is only wines poor in alcohol that turn viscous. A red wine which is rich in tannin is seldom affected.

Hartmann cultivated in a pure state an organism derived from a dry yeast mass in Java, mainly consisting of rice starch named by him *Torula colliculosa*. The size of the cells varies from 1.7 to 9.7  $\mu$ . It forms a smooth and moist glistening surface on wort-agar, but within twelve to fourteen days numerous eruptions appear about the size of a pin's head. In these are to be found the large cells. The cells of a young culture in the absence of these points cannot ferment maltose, but the large cells ferment it fairly strongly. This *Torula* ferments saccharose, glucose, raffinose, and levulose. By long-continued inoculation in unhopped wort this *Torula* can adapt itself to the vigorous fermentation of maltose. Purely cultivated *Torula* transferred to wort-agar from the eighth fermentation was only able to ferment maltose when the points had been reproduced.

Adametz, in conjunction with Winckler, found two *Torulas* in Olmützer Quargel cheese, one of which develops a yellowish-green fluorescent colouring matter on nutritive gelatine, and attacks the lactose, forming carbon dioxide but no alcohol.

In kephir, Freudenreich found a *Torula* (*Sacch. kefir*) along with three species of bacteria, which played a part in the fermentation. It is small (3 to 5  $\mu$ ) and oval, gives no fermentation in milk, but a peculiar yeasty taste. When the milk has been hydrolysed, a process carried out by the *Streptococcus* present in kephir, a fermentation takes place. Dextrose and maltose are both fermented.

The yeast fungi which occur in strong salt solutions occupy a singular place. Thus Wehmer has described a "salt yeast" which occurs in large numbers in pickled herring. It is a small, round, or oval *Torula*, which thrives well in nutritive solutions containing from 10 to 15 per cent. of sodium chloride, and it remains capable of development for weeks and months in presence of 24 per cent. It is probably derived from sea water or from the fish themselves, and brings about, according to Wehmer, the formation of trimethylamine in pickled herring.

In an examination of the ling organism (*Torula epizoa*), K. Höye was led to undertake a series of air analyses along the coast of Norway. He utilised as a substratum a wheaten flour paste to which 17 per cent. of sodium chloride had been added, in order to prevent the growth of the usual moulds and bacteria. On this salty substratum a few budding fungi developed, one of which gave spore-formation (described under the *Saccharomycetes*); the other two are *Torulas*.

*Levure de Sel*  $\beta$  is a roundish oval yeast, the shape of which depends on the amount of salt present. By the addition of 15 per cent. of salt the cells are more oval than in the presence of smaller quantities, but with 20 per cent. many are distinctly pointed, somewhat like a carrot. Short fine points project from the membrane of the cells, two or three on each. The cells are sometimes connected by these threads. It does not develop in cider.

*Levure de Sel*  $\gamma$  is very variable in shape. It grows like *Monilia* in fish broth containing 15 per cent. of salt. It forms oval cells in chains in presence of 25 per cent., and gives absolutely round cells with 35 per cent. of salt. There is no growth in cider.

Amongst beverages or foods prepared with a large per-

centage of salt (6 to 17), in which yeasts play a part in the preparation, the Japanese Soja (Shoju), Japanese bean broth (Miso), which contains 1.92 per cent. of alcohol; and Javan bean broth (Tao-Tjiung) may be mentioned. In the first case *Saccharomyces* are found (see description); no description has yet been given of the yeast species occurring in the last two cases.

According to Wehmer the sauerkraut fermentation is not simply a lactic acid, but is always accompanied by an aleoholic fermentation. He states that three budding fungi occur with morphological differences, which he calls *Sacch. brassicar* I., II., III., but he adds that no spore-formation takes place, so that we are really dealing with *Torulas*. The small species No. I. has an elongated spherical shape, No. II. is spherical, and No. III., which is found most frequently, is ellipsoidal. The yeasts are said to be the cause of foaming. By destroying the sugar residues which have not been attacked by the lactic bacteria, they are of value in enhancing the keeping qualities of the preserved food. According to R. Schulz, yeasts of the type of *S. ellipsoideus* and *S. apiculatus* are present in the souring of beans, certainly in preserved raw beans. Wehmer states that the fermentation proceeds with the formation of gas.

According to T. Inui, a drink called Awamori is prepared on the Luchu Islands in the fermentation of which a *Torula* takes place. The cells are at first elliptical, and then round. In wort they are elliptical, and produce about 6 per cent. of alcohol. The propagation of the cells only ceases in presence of 20 per cent. of alcohol.

These forms are closely allied to the red budding fungi (the "pink yeast" of medicinal bacteriology) which are universally distributed in atmospheric dust. Most of them bring about no fermentation; many occur as film yeasts.

*Torula b* = *Torula mucilaginosu* n.sp. The cells are oval and somewhat larger than those of *Torula a* (see p. 388) with a length of 5 to 5.6  $\mu$  and breadth of about 2  $\mu$  (Fig. 93).

By sowing in wort a slight turbidity takes place at first, and almost immediately afterwards a slimy yeast ring forms on the side of the flask with a dirty red colour, together with

a slight slimy sediment, which is only visible on shaking. The ring increases in thickness, and grows towards the middle of the flask, so that this is gradually filled from top to bottom with a rose-coloured slimy mass. A true sediment is not formed. The clots of slime which fall from the ring form a more or less thick layer on the bottom. On taking out the sample the liquid is quite ropy. No fermentation takes place with dextrose, maltose, lactose, saccharose, raffinose, and dextrin. Saccharose and raffinose are inverted. By the cultivation of this species in wort with varying quantities of alcohol, a small yeast ring is observed in eight days at 25° C. in wort containing 1 per cent. of alcohol. The ring is not formed in presence of 2 per cent., and no development whatever takes place in presence of 5 per cent. of alcohol.

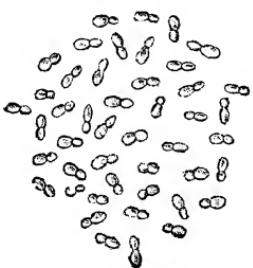


Fig. 93.—*Torula b.*—Young culture (Brask).  $\times 500$ .

To determine how far the slime formation is influenced by the proportion of sugar and albumen, the following experiment was carried out. In a pure dextrose solution the development was poor (1-5-10-20 per cent. dextrose), and no yeast ring formed, only a minute sediment. By the addition of peptone the ring formation is restored. When the quantity of sugar remains constant

(10 per cent. dextrose), while that of peptone rises from 0.1 to 0.2, 0.5, and 1 per cent., the ring formation is favourably influenced. With a constant quantity of peptone (0.5 per cent.) and increasing quantities of dextrose (5-10-20 per cent.) the slimy ring formation is reduced with increasing quantity of sugar. This shows that the slime formation depends upon the presence of albuminoids, and not upon that of sugar. The surface colonies on wort-gelatine (10 per cent.) are round, moist, and glistening, pale pink in colour, and slightly arched. The young colonies have smooth edges, the older show a depression in the middle and slight transverse furrows at the edge.

*Torula c* = *Torula cinnabarina* n.sp. The cells are predominantly of an elongated and oval shape, often provided

with promycelium. The length varies from  $7.7$  to  $10.5\ \mu$ , breadth from  $3.5$  to  $5.0\ \mu$ . Giant cells often occur, sometimes with rather elongated form,  $14.6\ \mu$  in length, sometimes almost spherical and  $9.5\ \mu$  in diameter (Figs. 94 and 95).

When sown in wort or in various sugar solutions, it first forms a smooth and afterwards a dry wrinkled film with intense crimson-lake colour. The liquid under the film is clear. No sedimentary yeast is formed, and no fermentation phenomena can be observed. The wort undergoes a remarkable bleaching effect in older cultures. At  $25^\circ$  isolated islands of film appear on the surface in sixty hours, and few cells show indications of promycelium. The formation of promycelium takes place freely in eighty hours. The formation



Fig. 94.—*Torula c.*—Young culture (Brask).  $\times 500$ .

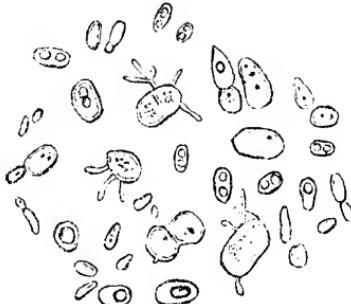


Fig. 95.—*Torula c.*—Film-formation, old culture (Brask).  $\times 500$ .

of buds takes place both on the promycelium and on the cells. There is no fermentation in dextrose, maltose, lactose, saccharose, raffinose, or dextrin, but saccharose and raffinose solutions are inverted.

In wort with 1 to 2 per cent. of alcohol a feeble fermentation is visible. With higher percentages of alcohol, no development takes place. The surface colonies on wort-gelatine (10 per cent.) are round, pale pink in colour and opaque. At a later stage the surface is warty, the edge jagged. The old colonies are dry, and display a network of furrows and a finely fringed edge.

Jansson and Mertens described a red form appearing in English beer, the cells of which develop many buds at one

and the same place on the mother-cell—Will's "crown" formation. They form promycelium in the film where long mycelial threads are also to be found. L. van den Hulle and H. van Laer found a red *Torula* in Lambic (a Belgian beer), which decolourised wort, and imparted a bitter taste to it. Will found a red species on green malt. When dried the malt assumed a dirty-brown colour. Kilned malt was discoloured and not presentable. The infection was derived from steeping water.

Fischer and Brebeck's *Blastoderma salmonicolor* has a promycelium which divides off into pear, plum, and kidney-shaped cells. This species forms a tough and very wrinkled film; it is found in sea water.

Lasché has described two red *Torulas* under the names *Mycoderma humuli* and *M. rubrum*. The first is found on hop leaves, and forms a film on nutritive liquids. Oval, sausage-shaped, or, to a great extent, irregular cells frequently form a promycelium, from which the buds divide. Gelatine is rapidly liquefied. This species gives no fermentation, and cannot develop in beer. *M. rubrum*, derived from a chance infection on a gelatine plate, has oval or sausage-shaped cells, often linked in short chains. Promycelium formation occurs more frequently, and this species develops longer in wort, whilst the colour of the film is a lighter red. It gives no fermentation, and does not develop in beer.

Red *Torula* species occur in milk and cream, where they often form red specks on the surface (air infection), as well as in butter and cheese. Demme declares that the species occurring in milk and cheese is the cause of catarrh of the stomach in children.

Kramer found a top-fermentation *Torula* in must which produces a red soluble colouring matter. It ferments dextrose, and produces 4.5 per cent. by volume of alcohol in a 10 per cent. solution. Saccharose is inverted by this species, and maltose is directly fermented. Lactose, on the other hand, is not affected.

The "black yeasts" are probably related to *Dematium*, *Cladosporium*, or *Fumago*. They have been found and described by Marpmann, Grotfert (on cheese), and Guilliermond (on carrots).

**Torula Yeasts fermenting Lactose.**

Duclaux found a yeast-fungus in milk which induces alcoholic fermentation in a solution of lactose. This fungus appears to be most closely related to the *Torula* species. The cells are 1.5 to 2.5  $\mu$  in diameter, and almost spherical. According to Duclaux's experiments, this yeast is more aërobic than the ordinary alcoholic yeasts. Even with strong aeration of the liquid, the whole of the lactose is used up in the alcoholic fermentation. In a 5 per cent. solution of lactose 2.5 per cent. of alcohol was formed in eleven days at 25° C. The most favourable temperature for the fermentation of a neutral solution is 25° to 32° C., whilst at 37° to 40° C. the fermentation ceases. Small quantities of acid have a retarding influence on the fermentative activity of this yeast.

Adametz likewise describes a budding-fungus which ferments lactose ("*Saccharomyces lactis*"). Since this fungus does not yield endogenous spores, it must be classed in the group of *non-Saccharomycetes*. The cells are of about the same size as those of brewery yeasts, and are spherical and elliptical. The colonies grown on peptone-gelatine are round, with slightly jagged borders, and are of a dark brown colour. A stab-culture in wort-gelatine yields a dull, flat mass on the surface and a vigorous growth in the puncture channel, and from this numerous offshoots penetrate into the gelatine. In sterilised milk this fungus induces fermentation phenomena within twenty-four hours at 50° C., in forty-eight hours at 38° C., and in about four days at 25° C. In this fermentation the lactose alone is decomposed.

Both of the species mentioned above have been more closely investigated by Kayser, together with a new species, which likewise ferments lactose, and belongs to the *non-Saccharomycetes*. All three yield colonies on gelatine, which are more widely spread than those of beer- and wine-yeasts; in the middle of the colonies there is a thick portion, while the border resembles mycelium. In milk and in neutral liquids, when sufficiently aërated, they induce an appreciable fermentation at 25° to 30° C. The milk does not coagulate or become viscous during the alcoholic fermentation. All three species

ferment lactose, galactose, cane-sugar, glucose, invert-sugar, and finally maltose, but the last only with great difficulty. In the fermentation of milk-sugar with these yeasts, the resulting liquids are as rich in alcohol as the strongest beers. Kayser remarks that it may, perhaps, be possible to make practical use of this observation and by means of these fungi convert the large quantities of whey, obtained in the manufacture of cheese, into an alcoholic liquor.

Beijerinck has described two yeasts which also ferment lactose, and which must be provisionally regarded as *non-Saccharomycetes*; these are *Saccharomyces Kefyr*, which occurs in kephir-grains and consists of longish cells of varied shape, and forms slightly jagged colonies liquefying gelatine; and *Saccharomyces Tyrocola* (from Edam cheese), which consists of small roundish cells, and forms snow-white colonies on gelatine. Beijerinck found that these two species secrete a particular invertive ferment (*lactase*) which inverts not only cane-sugar but also lactose; it does not, however, invert maltose. It is stated that lactase may be prepared as follows: —A 5 per cent. solution of lactose, containing nutrient salts and asparagin, is fermented with kephir-yeast; the product is filtered and the ferment is precipitated from the filtrate by the addition of alcohol. According to Schuurmans Steckhoven, however, the enzyme of Beijerinck's kephir-yeast does not invert lactose.

In Lombardy Grana cheese a unilaterally budding top-fermentation yeast was discovered by Bochicchio, which is called *Lactomyces inflans caseigrana*. The growth consists of round, ellipsoidal, and oblong cells, and forms whitish colonies on gelatine, with smooth edges. No spore-formation was observed. In lactose-broth it produces a vigorous fermentation at 25° to 40° C.; the best temperature for the development is about 30° C., the limit of existence at about 60° C. Whey infected with this species is converted into a foaming beverage of a somewhat agreeable taste.

Weigmann has isolated a pure culture of *Torula* from a defective butter. By fermentation in milk 51.2 per cent. by weight of alcohol and 34.4 per cent. of carbon dioxide, together with 3.6 per cent. of butyric acid were produced. Orla Jensen

has also isolated a *Torula* from butter which fermented maltose in addition to lactose. P. Mazé found ten different *Torulas* in soft cheese, one of which fermented lactose only; the others, in addition, fermented dextrose, levulose, maltose, and saccharose. The fermentations are more rapidly carried to an end, and a higher yield of alcohol is obtained if they are carried out in an alkaline liquor. Martin bouillon makes a good substratum with 0.088 per cent. of sodium carbonate. Mazé believes it to be probable that these species produce aromatic bodies in soft cheese. In American cheese and milk a *Torula* occurred at one time, producing a bitter taste. Harrison proved that the infection was derived from milk cans, which, in their turn, had been infected by exposure under maple trees to dry air. The yeast-fungus, named by Harrison *Torula amara*, gives a strong and unpleasantly bitter taste to milk in fourteen hours at 37° C.: fermentation is brought about, and an odour developed resembling that of plum kernels; the flavour becomes more astringent. At a later stage the milk curdles somewhat, and possesses a slightly acid and ethereal aroma. Lactose, glucose, and saccharose are easily fermented. In milk the last trace of sugar is fermented. The organism grows in broth containing 2 to 4 per cent. of lactic acid.

A complete and comprehensive description of these lactose-fermenting *Torulas* (together with the lactose-fermenting *Saccharomyces*) is given by Heinze and Cohn. They undertook a special and very detailed morphological and physiological investigation into Adametz' *Sacch. lactis* and Beijerinck's *Tyrocolta*.

Kalantha detected three lactose-fermenting species in Mazun—viz., the greenish Mazun yeast with giant colonies, which are first greenish-grey and then plum-red, and two others, which, however, are declared by P. Lindner to be identical with the first. They ferment lactose, saccharose, trehalose, dextrose (feeble), but neither maltose nor  $\alpha$ -methyl-glucoside; they also produce acid.

*Torula* species have also been detected in many defective butters and cheeses, which appear to be of more or less importance, thus Rogers found a *Torula* in a fishy and rancid

butter. It occurred several times in cases of preserved butter, and contains a fat-cleaving enzyme. Adametz has observed a *Torula* during the blistering of cheese, and it was also discovered by Bochicchio in Lombardy Grana cheese. The *Lactomyces inflans caseigrana* alluded to above brings about a marked blistering on the outer parts of hard cheese. It coagulates sterile milk, and partially liquefies the coagulum without noticeable formation of acid. It must, therefore, contain a clotting enzyme and a tryptic enzyme.

***Saccharomyces apiculatus* Reess. (Fig. 96.)**

According to our present views, the name of this ferment is incorrect, for only those budding-fungi which yield endogenous spores are considered to belong to the *Saccharomycetes*, and the fungus in question does not possess this property. We will, however, provisionally retain the old generic name, as has been done by Hansen, until systematic classification has been further developed.

This ferment was the subject of one of the finest and most thorough biological investigations of our time, for Hansen was enabled, after several years' work, to determine both its habitat in nature and its regular migrations at different seasons of the year (p. 272). The reason why this species was selected for the investigation was that, while other species occur in very varied and uncertain forms, making the study of their occurrence in different localities very difficult, this ferment can be recognised with certainty, for it always occurs in cultures with lemon-shaped cells; this is the typical form of the species.

*S. apiculatus* occurs abundantly in wine fermentation, especially during the early stages, and also in spontaneously-fermented Belgian beer (Lambic, Faro, Mars, Kriekenbier), and, according to van Laer, imparts to it its peculiar taste and odour. In nature it is found on ripe, sweet, succulent fruits.

If a little of such a growth is examined under the microscope in a drop of nutritive liquid, the development of the fungus can be followed. This is very characteristic (compare Fig. 96). It is seen that the buds formed from the typical

lemon-shaped cells may be either lemon-shaped (*a*, *b*, *c*, *e*, *f*) or oval (*a-c*) ; it will also be noticed that the oval cells must first form one or more buds before they are able to assume the lemon-shape (*e-f*), and finally, that the lemon-shape of a cell attained by budding (*k*, *k'*, *k''*) may be lost again on the

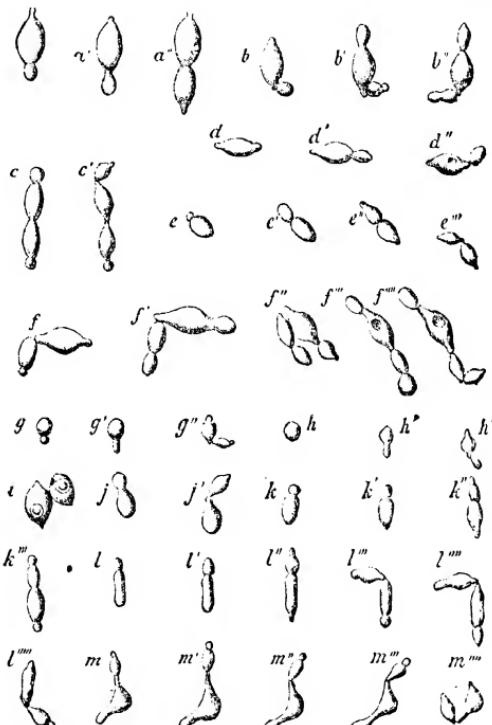


Fig. 96.—*Saccharomyces apiculatus* (after Hansen).—Budding cells: *a-a'*, a cell which in the course of 34 hours developed a bud at its lower extremity; *b-b'*, a similar series, showing the development of a bud at the upper extremity of the mother-cell, whilst a bud had been previously formed at the opposite end; *c* is a chain of cells, *c'* is the same three-quarters of an hour later; the lowest bud had assumed the typical form of the species like those above it, but in the figure it is seen from the end, so that its longitudinal axis is at right angles to the plane of the paper; *d-d'*, development during 1½ hours; *e-e'*, during 2½ hours; *f-f'*, during 3 hours: in *e-f* it is seen that the oval cells first develop a bud and only subsequently assume the typical lemon-shape; *g-m*, abnormal cells: progressive development.

formation of a new bud (*k'''*). Under other conditions the cells may assume quite different forms, sausage-shaped, crescent-shaped, like bacteria, etc. (*g-m*). Does any rule govern this apparent confusion ? It has just been shown that the fungus

can form two kinds of buds, and that the oval buds must develop one or more new buds before they can assume the typical form. The question then is : Under what conditions are those two kinds of buds developed ? It was shown by means of culture experiments that the lemon-shaped buds are developed especially during the early stages of the culture, but are afterwards crowded out by the oval forms.

A further description of the fungus from a physiological and biological standpoint will now be given.

*Sacch. apiculatus* is a bottom-fermentation yeast, capable of exciting alcoholic fermentation in beer-wort ; the fermentation in this liquid is, however, a feeble one, only 1 per cent. by volume of alcohol being produced, whilst a bottom-yeast under the same conditions gives 6 per cent. This arises from the fact that *Sacch. apiculatus* cannot ferment maltose. Hansen also found that it does not secrete invertase. On the other hand, it excites a vigorous fermentation in 15 per cent. and 10 per cent. solutions of dextrose in yeast-water, and in one experiment as much as 3 per cent. by volume of alcohol was formed. After three months the liquid still gave the sugar reaction whilst the amount of alcohol had not increased during the last six weeks. The fungus was thus unable to complete the fermentation. In another of Hansen's experiments as much as 4.3 per cent. by volume of alcohol was produced.

According to Röhling, the application of oxygen increases the formation of alcohol by 5 to 8 per cent., both the life energy and the power of resistance of the cells being greatly increased. Without oxygen, alcohol formation amounted to 2.3 to 3 per cent. The cells are very sensitive to chemical reagents. Sulphurous acid (0.025 per cent.) almost entirely prevents its fermentative activity (sulphuring casks), and alcohol acts very restrictively. On the other hand, tannin only acts at a strength of 0.5 per cent.

It was found from experiments, in which a mixture of this fungus with *Saccharomyces cerevisiae* was grown in beer-wort, that it was crowded out by the latter, being the weaker species, although it retarded the growth of *Sacch. cerevisiae* to no small degree.

In flasks with the same beer-wort, and at the same tempera-

ture, each containing one species, *Saccharomyces apiculatus* will multiply to a greater extent than the brewery yeasts in a given interval of time.

At the critical time of the year, the ferment, if present in the wort in considerable quantities, may exist for a length of time side by side with brewery yeasts, and will no doubt retard its action a little; but when the beer is transferred to the lager cellar, the fungus remains inactive in the alcoholic liquid, and frequently perishes.

According to Will, the fungus frequently occurs in slight traces in low-fermentation beer-yeast; it may be caused to multiply more freely by treatment with tartaric acid.

Will mentions a case in which the yeast in a brewery in Baden was so strongly infected with *apiculatus* that these could be directly detected by their characteristic form, and, indeed, were seen in large numbers. In the cask store and in the deposit from diseased beer, cells of *S. apiculatus* are occasionally found in considerable quantity in a living condition, along with wild yeasts, and the flavour of the beer may then be influenced.

Müller-Thurgau and Wortmann regard the fungus as injurious to wine, for it not only directly prejudices the quality of the wine and must, but also checks fermentation, and thus gives rise to disease.

The organic non-volatile acids (tartaric and malic acid) present in fruit juices and grape must disappear by the cultivation of *S. apic.* It is possible that they serve as sources of carbon for yeast, or else that they are decomposed in the fermentation process. As fast as the acid is consumed a fresh formation of acid takes place.

Meissner has shown that *S. apic.* can produce lactic acid; succinic acid is also produced. According to P. Lindner, fruit ethers are formed. Will found that certain species cause the production of a bouquet resembling amyl ether: others give a fusty smell. According to Schander the pronounced "Böckser" taste occurring in wines may be produced by certain species of *S. apic.* Proteolytic enzymes are present, and gelatine is readily liquefied by the organism.

Many varieties or races of *S. apic.* are known. This was

first observed by K. Amthor. One race produces more volatile acid or more alcohol and glycerine than another.

Müller-Thurgau subjected seven different races to examination. They produced from 2.5 to 3.8 per cent. by weight of alcohol in grape juice; two races gave as much as 6 per cent. Schander found cells of different shape and size in the *apiculatus* yeasts that he examined, some short, thick, and lemon-shaped, and others thin, elongated, and of a less distinct lemon-shape. Röhling's different species exhibited slight physiological differences, and Will, who isolated a few species from wort, showed that they can be distinguished by the different aroma produced in wort.

P. Lindner has described *Sacch. apic.*, var. *parasiticus*, living on wood-lice. One end of the cell is often drawn out to a point which penetrates their eggs and then forms a bud. In this way the eggs infect the mother insect, and the yeast is distributed by their offspring. They cannot be cultivated in fruit juice or in artificial nutritive liquids. We are dealing here with an obligatory parasite.

The question how far *S. apic.* is capable of forming endogenous spores was thrashed out long ago; in 1894 it was answered in the affirmative by Beijerinck. He stated:—"If a convincing proof is required of the property of spore-formation, it is only necessary to isolate this yeast when conveyed by air or dry dust on to fruit. In this way cultures are occasionally met with, containing individual cells swollen to asci, with from four to six ascospores." He did not, however, observe a germination of the spores.

P. Lindner examined cells of *S. apic.* from blossoms of *Robinia Pseudacacia* in streak-cultures with wort contained in moist chambers, and found a development of spores in the culture. They show a distinct wall and a granulated protoplasm. According to the picture, each cell contains only one spore. Again, no germination of spores was observed. Descendants of this growth showed no tendency to form spores.

Influenced by these experiments, Lindner, classed the *apiculatus* yeast as a new genus, which he named *Hansenia*. A. Röhling worked with pure cultures of races derived

from samples of soil, and succeeded in bringing about spore-formation by cultivation on gypsum blocks for eight to ten days (the temperature is not mentioned). He also succeeded in causing the spores to germinate in an extract of horse dung with 5 per cent. of grape sugar. It may also be mentioned that J. C. Holm detected lemon-shaped cells, somewhat larger than the ordinary *apiculatus* cells in certain fermented ciders from England, in the author's laboratory in 1894. By transference to gypsum blocks after a single cultivation in dextrose-yeast water, isolated cells were observed with one or two spores. On account of the extraordinarily small number of spore-forming cells, it was impossible to carry out germinating experiments. By prolonged cultivation and transference of cells to gypsum blocks, to nutritive gelatine, or to small quantities of sterile water, he never again succeeded in bringing about spore-formation. During budding, a septum was observed between the mother- and daughter-cell. J. C. Holm assumes that this form is closely related to one of the *S. Ludwigi* (*Saccharomyces Ludwigi*), and that possibly the work just mentioned was carried out with such forms, and not with *S. apic.* Reess.

#### *Mycoderma cerevisiae* and *vini*.

It is characteristic of these species that they very readily form films on various alcoholic liquids. Under these names are included a number of different species, some of which may excite a feeble alcoholic fermentation; they behave differently towards lager beer, some causing disease whilst others do not.

The *Mycoderma cerevisiae* (Fig. 97) examined by Hansen, which is universally met with in Copenhagen breweries, forms variously-shaped cells. The cells are usually transparent and less refractive than the true *Saccharomycetes*; in each cell there are generally one, two, or three highly refractive particles, which often have a quivering, rolling motion. This micro-organism forms a dull, greyish, wrinkled film on wort and beer, and does not excite alcoholic fermentation; neither does it invert solutions of cane-sugar.

The colonies on the surface of the gelatine are light grey, dull, and spread out like a film or hollowed like a shell. By

means of this macroscopic appearance *Mycoderma* is readily distinguished from the ordinary *Saccharomyces*, which, on the same medium, form light greyish-yellow colonies with a dry or lustrous surface, and a more or less arched form. *Sacch. membranæfaciens*, which differs so markedly in its biological behaviour, and which very rapidly gives a strong film on the liquid, alone resembles *Mycoderma* in its behaviour on plate cultures.

This kind of film-formation was noted by Hansen when lager beer had been exposed in open vessels at temperatures between 2° and 15° C.; at 33° C. development still occurred,

but at temperatures above 15° C. this species gave place more and more to competing forms. As low temperatures are favourable to its development, it will readily thrive in the storage cellar, especially as lager beer forms a much more favourable medium for its growth than wort. This is seen to be the case when traces of a pure film are introduced into lager beer



Fig. 97.—*Mycoderma cerevisiae* from Copenhagen breweries (drawn from nature by Holm).

and wort, contained in open vessels, and then left to develop; the culture in lager beer nearly always remains pure, while in wort various other species make their appearance.

In Hansen's comprehensive experiments on Carlsberg beer, it was always found that both lager and export beers were attacked by this fungus; but there was never the slightest indication that the beer had acquired any disease from this source. The fungus was widely distributed just at those periods when the beer was found to be particularly stable and of good flavour. This has also been confirmed by numerous experiments on lager and export beers conducted by Grönlund and A. Petersen, and those carried out in the author's laboratory. It is self-evident that we are only speaking of beer which has been properly treated. In imperfectly closed bottles and casks, *Mycoderma cerevisiae* will of course rapidly

develop a film, which is sufficient, unaided, to destroy the product.

Béthounek was the first to observe that, under certain conditions, *Mycoderma* may cause considerable injury in the brewery. Subsequently, Kukla described a curious cloudiness in lager beer, having the appearance of a cloud of fine dust in the liquid, which manifested itself either during storage or after tapping ; he attributes this disease to *Mycoderma*, and further assumes that it is weak wort, having certain peculiarities in its composition, which specially favours the development of *Mycoderma*. It is to be hoped that further investigations will throw more light on this subject.

Hansen expressed the opinion that the name *Mycoderma cerevisiae* denotes not one, but several different species, and Lasché's experiments subsequently confirmed this. The latter investigator describes four different species which he isolated from cloudy beers. They are distinguished from the species described by Hansen by the fact that they produce alcohol in beer-wort ; one yields 0.26 per cent. by volume, two yield 0.79 per cent., and the fourth produces 2.51 per cent. Lasché concludes from his experiments that these four species cause diseases in beer, both turbidity and changes in taste and odour ; in this respect they also differ from Hansen's *Mycoderma*. Lasché is inclined to assume that the chemical composition of the wort has no influence on the disease caused by *Mycoderma*, for, in his experiments, the disease was produced in worts of high extract and worts of low extract, in worts rich in sugar and worts poor in sugar.

Winogradsky found that the *Mycoderma* occurring in wine, prepared in pure culture by Hansen's method, alters its shape with the composition of the nutritive solution ; he experimented both with solutions, the mineral constituents of which remained constant while the organic substances varied, and also with solutions in which the reverse was the case.

Many experimenters have subjected *Mycoderma* to close investigation during the last few years, and especially Henneberg, Heinze, Meissner, Seifert, and Will. Meissner's researches are particularly comprehensive, both morphologically and physiologically, and they concern twenty-three different species

of *Mycoderma vini*. Greater or less distinctions were noted in the shape of the cells, their content in glycogen, the presence of oil drops, and in their giant colonies. The film-formation also showed distinct differences with different species, both with regard to the time required for their appearance, their character, and colour (white, cream, yellowish-brown, and yellowish-olive-green). When covered with the film, the liquor remains clear in certain cases, whilst in others a turbidity takes place of a permanent or temporary character.

Will believed that the decolorisation of the liquid may be referred to the formation of acid brought about by the film cells, whereas Heinze thought that it was caused by the removal of acid. Meissner confirmed the fact that a more or less marked decolorisation takes place, but showed that afterwards a reversal of the colour tints may come about, so that must which had turned pale assumed by degrees a dark brown colour. Not only is the total amount of acid destroyed, but the must at last acquires an alkaline reaction. Meissner succeeded in proving experimentally both with large and small amounts of must, that one and the same race may appear, first as a producer, and then as a destroyer of acid. It is, therefore, necessary to forego the division of film yeasts into acid-destroying and acid-forming species. Once the sugar in the must is destroyed, all so-called acid ferments will act as destroyers of acid. With regard to this question of the formation and destruction of acid, Meissner also states that when an increase of acid takes place it must be regarded as a result of two simultaneous processes of construction and destruction of acid, and that production has exceeded destruction. If, on the other hand, a reduction of acid takes place, the destructive action must be regarded as exceeding the constructive.

Butyric acid is formed amongst others, and ammonium compounds are also produced.

Meissner's experiments regarding the reaction of the *Mycodermas* with organic acids gave the following results:—Malic acid is only very slightly attacked by certain races, but strongly attacked by others. Tartaric acid is slightly decomposed. Lactic, citric, and succinic acids are in some

cases strongly affected. Acetic acid reacts strongly with a few species ; in other cases the species cannot grow at all in a solution containing acetic acid.

Alcohol, the sugars, glycerine, and tannic acid are decomposed. Alcohol is converted by an oxidation process into carbon dioxide and water, but may also act as an organic foodstuff. As early as 1878 Schulz found that "the film fungus can produce within itself ready formed organic compounds, and requires nothing but ammonia and alcohol for the purpose." Schulz did not, however, work with pure cultures.

Meissner utilised for his experiments both the nitrogenous nutritive material used by Schulz (ammonium nitrate, asparagin, ammonium tartrate) and an artificial solution containing ammonium phosphate and ammonium chloride, together with the necessary mineral constituents. The vigorous growth of *Mycoderma* proves that these two solutions supply them with nitrogen. Consequently the alcohol of the nutritive solution is partially respired and partially utilised for the building up of the cells. With regard to the sugars, Meissner found that the *Mycoderma* species cultivated on sterile grape juice respired dextrose and laevulose to some extent, partially producing acids from them. On artificial nutritive solutions which contain dextrose or saccharose as the only organic substance in addition to the necessary mineral nutrients, the sugars are oxidised, part being utilised for the construction of new cells, and part for the fresh formation of acid. Glycerine is not only destroyed, but may be produced from other organic substances. This fact was confirmed by W. Seifert.

The *Mycodermas* also produce various volatile acids. Thus Wortmann has drawn attention to the fact that many wines which have become filmy have an odour which resembles rancid butter to an extraordinary degree. Butyric acid has been formed in these cases.

Lafar found in a cask store a *Mycoderma* which imparted a flavour to beer resembling wine ether. Non-volatile acids and esters are also formed.

In finished wines the yeast has generally, but not always, finished its special work, but nevertheless wine may undergo

fundamental alterations caused by other micro-organisms, and amongst them, according to Wortmann, *Mycodermas* take the first place. By their agency, alcohol is converted into carbon dioxide and water, and they also influence the amount of acid and destroy the bouquet. A wine may become filmy, and may deteriorate in time without a visible covering of film appearing on its surface. The suspended cells of *Mycoderma*, which need not be numerous, may bring about in the course of years the same action which would occur more rapidly when the cells form a coherent mass on the surface. *Mycodermas* which often inhabit corks (like the moulds) may impart the well-known corked taste to wine.

Will has isolated a *Mycoderma* from top-fermentation beer, which brings about a marked decolorisation of beer. This occurs within a short time at the high temperatures requisite for completing top-fermentation. The species develops a large amount of acid in beer, but under certain conditions a destruction of acid may also take place. It cannot bring about alcoholic fermentation.

Will also made a series of observations concerning the duration of life in wort-cultures and in the dry state, and with regard to the power of resistance to heat in liquids. Seifert closely examined two *Mycoderma* species isolated from wine, which produced from 0.064 to 0.904 per cent. of acetic acid in an ordinary Austrian white wine, and reduced the amount of alcohol. The *Mycoderma* investigated by Heinze (*M. cucumerina*, Aderhold) was derived from a fermentation of sour cucumbers: he declares it to be a dangerous enemy of lactic acid fermentation. In beer the organism attacks alcohol strongly, and produces a bitter flavour. It is capable of producing alcoholic fermentation in dextrose solutions. In cider, with 10.62 grammes of sugar, it yielded 4.34 grammes of alcohol per 100 c.c. of fermented liquid, in five minutes, at 25° C. There is no fermentation with maltose, saccharose, and lactose. Heinze also closely investigated the question of acid production and acid destruction.

Henneberg mentions two species of *Mycoderma*, which he frequently found in distillery and pressed yeast. The shape of the cells is very varied. The one species frequently

forms *Mycelial* chains, and the other *Monilia*-like chains. The difference between the two forms is specially marked in cultures on solid substrata (giant-cultures, streak-cultures, etc.). In dextrose and laevulose solutions bubbles of carbon dioxide form under the film, and a fairly vigorous fermentation is produced by the cells, which sink to the bottom. Both species produce acetic ether. As is the case with *S. anomalous*, the optimum temperature for growth lies between 32° and 41° C. Dextrose and laevulose are readily fermented, galactose less readily, only traces of maltose and dextrin are fermented, whilst lactose, saccharose, raffinose, and inulin are not fermented at all. The two species can readily utilise lactic acid as food, and withstand up to 5 per cent. of the acid. Similarly they can withstand large quantities of alcohol (11 per cent.). The alcohol in this case is fairly quickly converted into carbon dioxide and water. These species are not, as might be supposed, capable of withstanding large quantities of organic acids in general, as was proved by a few experiments with increasing quantities of acetic acid in beer. In Egyptian Leben (Leben raib), Rist and Khoury found a *Mycoderma* about which they say that it is improbable that it has any particular influence on the special flavour of the Leben, but in any case the rapid development of a sharp acid taste, which renders the beverage undrinkable in a few days, must be ascribed to this organism. It forms both non-volatile acids and acetic acid. It grows excellently in glucose and maltose, and gives a fermentation with the former, whilst it converts glucose into acid, and brings about the combustion of alcohol. In lactose solution it gives no fermentation, only film-formation.

Although de Seynes, Reess, Engel, and Cienkowski claimed to have found ascospores in *Mycoderma*, it has since proved impossible to bring about their formation. It would appear from the drawings given that the fat globules, which occur in many unicellular fungi during the resting stage, had been mistaken for spores; in some cases the mistake appears to have arisen through the presence of an admixture of true *Saccharomyces*. The old name *Mycoderma* is, therefore, more appropriate to this fungus than the new term *Saccharomyces*.

## CHAPTER VI.

THE PURE CULTURE OF YEAST ON A  
LARGE SCALE.

## Industrial Application.

By the industrial application of pure cultures of systematically selected yeasts inaugurated by Hansen and the author, it became possible to carry out fermentations with certainty in a way that was impossible so long as an unknown yeast-mass was used containing not only a mixture of culture yeasts, but also wild yeasts, bacteria, and even moulds in certain cases. Such selected races can be preserved by appropriate means for a long time as small cultures which can be developed afresh into mass cultures.\*

One very important result of the adoption of the process was to prove that the visible fermentation phenomena do not in general give any insight into the purity of the fermentation. On the other hand, these phenomena may sometimes give valuable information regarding the condition of the yeast, which is directly connected with the nature of the nutritive liquid.

A real knowledge of the purity of fermentation can only be gained by a biological analysis combined with a microscopical examination.

Pasteur demonstrated the harm that bacteria can do when they develop in alcoholic fermentation, and at the same time he emphasised the importance of the oxidation of the nutritive

\* The important point in preparing a pure culture is the selection of the right species; not the purely mechanical isolation of an individual cell. It demands insight into the particular branch of the industry concerned. The point to bear in mind in preparing such cultures is that a yeast actually in use in the industry must form the starting point.

liquid for yeast activity. Hansen experimentally proved that some of the most dangerous diseases of beer are caused by wild yeasts.\*

When the absolutely pure culture developed in flasks in the laboratory has attained certain dimensions—*e.g.*, 1 to 2 litres of fluid yeast—it is ready for practical application. In the vast majority of cases the pure culture is further developed in a couple of small vats in which successive quantities of the nutritive medium are added in each case as soon as a vigorous development has taken place—*e.g.*, from 5-50-100-300 litres or from 5-50-500 litres, etc., depending on whether the yeast is a low- or a high-fermentation species. As a rule, the chief point to observe is that the mass of cells should be brought as quickly as possible to development, until the requisite quantity is secured for carrying out the normal fermentation on a large scale. In the preliminary stages the same nutritive liquid must be used as in the large fermentations. It will be obvious that during such a rapid development the specific character of the race of yeast will not be brought out. If it is desired to observe this during the small fermentations, they must be carried through to completion. If a regular supply of absolutely pure yeast must be kept in stock, it is necessary to use the pure propagating apparatus designed by Hansen and by A. Kühle.

The apparatus (Fig. 98) consists of three chief parts and the necessary connecting tubes. First, the air apparatus, with air pump (A) and air holder (B), secondly, the fermenting-cylinder (C), and, thirdly, the wort-cylinder (D). The air, which has previously been partially purified, is pumped into

\* The "natural" selection of yeasts proposed by Delbrück must not be confounded with the preparation of a single pure race. His process consists in subjecting the whole impure yeast-mass to a treatment which may consist of the application of a higher fermentation temperature, or pumping into a new vat after the appearance of foam on the surface, or pitching with wort from the first stages of fermentation, etc. A summary process of this kind will always yield an uncertain result, because the impure mass contains elements of very different character, and, even in the most favourable case, if by good luck the disease germs are restricted, it is evidently impossible to depend on securing the best-selected type of culture yeast. It is essential to isolate the yeast species and then to select those which best fulfil the stated requirements.

the receiver, and thence may be passed into either the wort or the fermenting cylinder. In either case the air is sterilised by means of a cotton-wool filter (*g, m*). The wort cylinder is directly connected with the copper from which the boiling hopped wort is run in; it is then aerated in the closed cylinder, and is cooled by spraying.

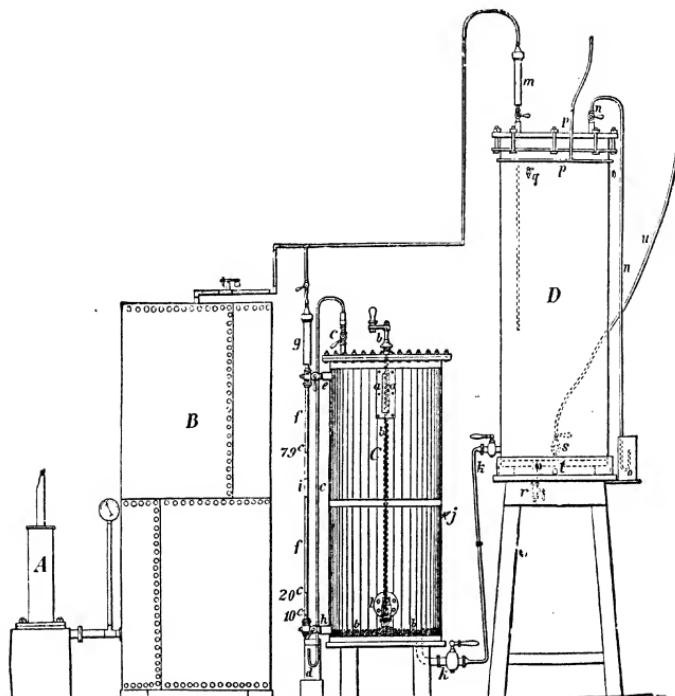


Fig. 98.—Yeast-propagating apparatus devised by Hansen and Kühle.—*A*, air-pump; *B*, air-vessel; *C*, fermenting-cylinder; *a*, window; *b*, *b*, stirrer; *c*, *e*, doubly-bent tube; *d*, vessel containing water; *l*, outlet cock; *f, f*, glass tube connected at *e* and *h* with the cylinder, and graduated for the measurement of fixed quantities of liquid; *g*, filter; *i*, rubber connection for glass tubes; *j*, tube with rubber connection for introducing the pure culture; *k*, *k*, connection with the wort-cylinder *D*; *m*, filter; *n*, *n*, doubly-bent tube; *o*, vessel containing water; *p*, *p*, spraying tube; *u*, connection with cock *s*; *t*, waste for cooling water.

The wort is then forced into the fermenting-cylinder, which, like the wort-cylinder, is constructed on the same principle as the ordinary two-necked flask. It is fitted with a doubly-bent tube (*c, d*), which dips into a vessel containing water; a vertical glass tube (*f, i, f*) for measuring the height of the liquid in the

cylinder; an appliance (*b*, *b*) for stirring up the deposited yeast, and a specially constructed cock (*l*) for drawing off the beer and the yeast. At about the middle of the cylinder there is a small side tube (*j*), fitted with india-rubber connection, pinch-cock, and glass-stopper. When a portion of the wort has been forced into the fermenting-vessel, the absolutely pure yeast—which is forwarded to the brewery in a flask specially constructed for this purpose—is introduced through the rubber tube at *j*; this is again closed, and the remainder of the wort may then be added either at once or after the lapse of a few days, according to the quantity of yeast introduced.

Where it is necessary to regulate the temperature during fermentation, the fermenting-vessel is surrounded by a water-jacket.

By means of this simple apparatus it is possible to obtain, at short intervals, absolutely pure pitching yeast, sufficient for about eight hectolitres of wort, and when once started the apparatus works continuously.

Another type of propagating apparatus has been described by Bergh and Jörgensen (Fig. 99). The filtered air passes through the three-way cocks at *A*, *B*, and *C*, into the two cylinders **A** and **B**. The upper cylinder holds about 50, the lower cylinder 160 litres. **A** is provided with a stirrer *E*, and a tube (*a*) for introducing the yeast and withdrawing samples. The bent tube *F* is an outlet for carbon dioxide. The tube *G P* connects the two cylinders, and the connection can be made or broken by means of the cock *G*. *H* is the outlet for water used in cleaning *A*.

The cylinder **B** is surrounded by a cast-iron jacket made in two parts; the upper portion serves as a water-jacket for cooling the wort and for regulating the fermentation; the lower portion is used as a steam-jacket, and is provided with a cock at *O* as an inlet for the steam, and another at *S* as an outlet. *M* is a ring-shaped tube provided with small holes; this is connected with the cold-water main during the cooling of the wort; the water is drawn off at *N*. The stirrer *J* is set in motion by means of toothed gear. The height of the liquid in the cylinder is indicated by means of a float, with pointer and arc *L*. A bent tube, *K*, projects from the top of

the cylinder. At the bottom is the cock  $Q$ , which is connected with the pipe  $b$  by cock  $T$ . Both the bent tubes dip into the vessel  $R$ , which is filled with water.

The wort is introduced into the lower cylinder, where it is treated in the ordinary manner. The pure culture is introduced into the upper cylinder, and is then washed down

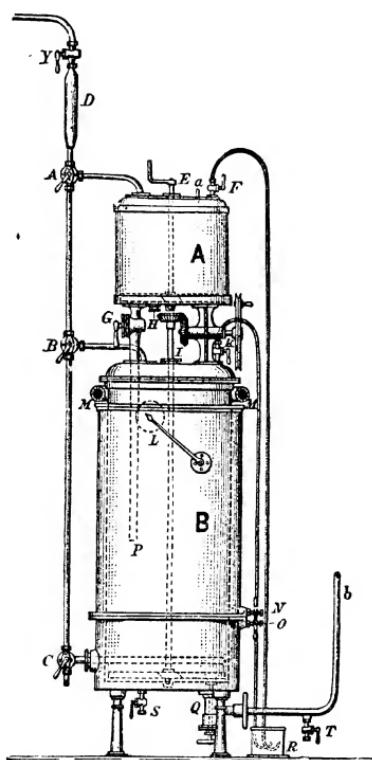


Fig. 99.—Yeast-propagating apparatus devised by Bergh and Jörgensen.

A comprehensive introduction to the method of dealing with the apparatus used in the laboratory for the preparation of pure cultures (moist chambers and flasks) is to be found, along with the mode of operating the two types of propagating apparatus, in a small hand-book of the author's, entitled *Practical Management of Pure Yeast*, London, 1903. Mod-

fications of both forms of propagating apparatus have been described by Brown and Morris, Elion, Thausing, Van Laer, Pohl and Bauer, Wichmann, Fernbach, Jacquemin, and others. P. Lindner and Marx have constructed a somewhat different apparatus.

In order to be able to send the selected pure cultures in a liquid condition to a distance, special forms of flasks were devised\* by Hansen (Fig. 100) and by the author (Fig. 101). The yeast can be sent a great distance in these flasks, and there is no difficulty in safely transferring it from the flask to the fermenting-cylinder of the propagating apparatus.

In sending small quantities of pure cultures, in such a manner that they may be safely and readily employed for further cultivation, the small Hansen flasks are employed

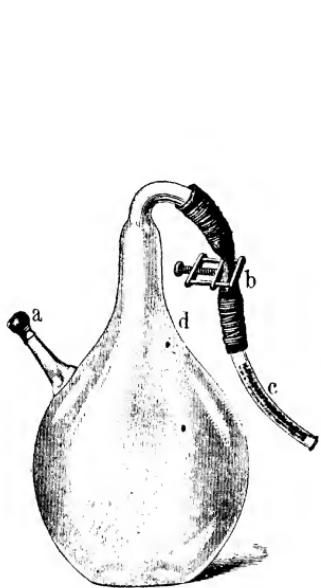


Fig. 100.



Fig. 101.

(p. 37). They are connected, in the flame, with the Pasteur flask in which the pure culture has developed. A trace of the yeast is transferred to the cotton-wool, and the flask is again closed in the flame with the asbestos stopper, which is then coated with sealing-wax. When the culture is to be used, the flask is again connected with a Pasteur flask containing wort, and the yeast is rinsed into the latter.

This process has made it possible to send large collections

of pure cultures to the most distant countries at a very small cost.

It is of the greatest importance to note that, even after the lapse of years, the particular yeast once selected can always be procured again, a sample of the pure culture being preserved in the laboratory in a 10 per cent. solution of cane sugar, kept in the flasks described in Chap. i. (p. 37), devised by the author for the purpose. Culture yeasts may be kept alive in such a solution for years without any alteration in their properties. It is of importance, in order that the culture yeasts may be kept unaltered for a long time, that the layer of yeast deposited upon the bottom of the flask should not be frequently shaken. During the introduction of a few drops into a Pasteur flask shaking can only be avoided by the use of the flask depicted in Fig. 9. With any other variety it is necessary to maintain a number of flasks for each species of yeast, and each one will only serve for a few infections. On the other hand, no effect of temperature has been observed during storage, and the dilution of the liquid can be avoided by the use of the two flasks constructed by the author, and shown in Figs. 8 and 9. All physiological laboratories concerned with fermentation possess such collections of preserved growths. The author's collection of species which have been gradually introduced into practice dates back to the year 1884, and numbers many thousands of specimens. A few of these species have retained those properties which are of industrial value for more than ten years. According to Hansen's and the author's experiments yeasts may be kept alive under such conditions for a much longer period.

Regarding the storage of dried yeasts on the large scale, A. Will has made extensive investigations, the results of which will be found in the technical literature.

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## INDEX.

## A

ACETIC acid, 268.  
 " bacteria, 92, 235.  
 " " influence on beer, 104.  
 " " influence on wine, 105.  
 " " Staining, 7, 95.  
 Acetone, 27.  
 Aération of wort, 280.  
 " yeast, 279.  
 Aërobic bacteria, 82.  
 Aëroscope, 60.  
 Agar, 50.  
 Air, Biological examination of, 59.  
 " Filtration of, 17, 62, 233.  
 " Hansen's investigation of, 65.  
 " Saito's 69.  
 " Sterilisation of, 23.  
 Albuminoids in yeast cells, 9, 228.  
 Alcohol, Amyl, 268.  
 " Ethyl, 267.  
 " Methyl, 268.  
 " as disinfectant, 28.  
 " -forming bacteria, 133.  
 Alcoholase, 249.  
 Alcoholic fermentation, Products of, 267.  
 Aldehyde, 268.  
 Ammonium fluoride, 27, 32.  
 Amœbوبacter, 80.  
 Amyl alcohol, 268.  
 Amylase in moulds, 256.  
 Amylobacter butylicus, 134, 135.  
 " ethylicus, 134.  
 Amylomyces ( $\beta$ ), 205.  
 " Rouxii, 203.  
 Anaërobic bacteria, 54, 82.  
 Araërobiosis, 240.  
 Analysis of yeast, 311.  
 Anatomy of yeast cells, 290.  
 Antiférmin, 27, 33.  
 Antigermin, 33.

Antinomium, 34.  
 Antiseptics, 24, 32.  
 " Accustoming fungi to, increasing doses, 30.  
 " influence on bacteria, 87.  
 " " on fungi, 178.  
 " Stimulating action of, 24, 174.  
 Apothecia, 186.  
 Ascococcus Billrothi, 150.  
 Ascogonium, 193.  
 Ascospore formation, 302.  
 Ascus, 169.  
 Aspergillus batatae, 197.  
 " flavescens, 182.  
 " flavus, 194.  
 " fumigatus, 182, 194.  
 " glaucus, 182, 191.  
 " niger, 174, 194.  
 " Oryzae, 195.  
 " repens, 174.  
 " Wentii, 197.  
 Asporogenesis, 284.  
 Autoclave, 15.  
 Auto-fermentation, 269.  
 Awamori, 395.

## B

BACILLE amylozyme, 134, 135.  
 Bacillus acidi lactic, 107, 109.  
 " acidificans longissimus, 108,  
 " 120.  
 " boocopricus, 129.  
 " Buchneri, 120, 125.  
 " Bulgaricus, 140.  
 " butylicus, 134.  
 " butyricus, 127.  
 " casei, 109.  
 " caucasicus, 136.  
 " coli communis, 111.  
 " cyanogenus, 114.

Bacillus *cyanofuscus*, 116.  
 " *Delbrücki*, 108, 120.  
 " *ethaceticus*, 134.  
 " *Fitzianus*, 133.  
 " *fluorescens liquefaciens*, 115, 151.  
 " *fluorescens putidus*, 132.  
 " *fœtidus lactis*, 115.  
 " *gummosus*, 144.  
 " *Hayducki*, 120, 125.  
 " *lactis aërogenes*, 145.  
 " " *pituitosi*, 145.  
 " " *saponacei*, 113.  
 " " *viscosus*, 114, 145.  
 " *Leichmanni*, 125.  
 " *Lindneri*, 122.  
 " *Listeri*, 125.  
 " *lupuliperda*, 132.  
 " *luteus*, 153.  
 " *Megatherium*, 151.  
 " *mesentericus vulgaris*, 143.  
 " *nobilis*, 112.  
 " *orthobutylicus*, 130, 134.  
 " *panis fermentati*, 124.  
 " " *viscosi*, 143.  
 " *pneumoniæ*, 134.  
 " *prodigiosus*, 153.  
 " *putrificus*, 153.  
 " *rudensis*, 116.  
 " *saccharo-butyrificus*, 130.  
 " *Schaffieri*, 115.  
 " *subtilis*, 152, 175, 182.  
 " *thermophilus*, 86.  
 " *vasculorum*, 151.  
 " *viscosus*, 146.  
 " " *bruxellensis*, 147.  
 " " *sacchari*, 142.  
 " " *vini*, 142.  
 " *vulgaris*, 111, 151.  
 " *Wortmanni*, 125.  
 " *Zeidleri*, 100.

Bacteria, 76.  
 " acetic acid, 92.  
 " influence on beer, 104.  
 " influence on wine, 105.  
 " Aërobic, 82.  
 " Alcohol-forming, 133.  
 " Anaërobic, 54, 82.  
 " Butyric acid, 125.  
 " cell contents, 78.  
 " wall, 79.  
 " chemical composition, 81.  
 " colouring matter, 80.  
 " fermenting cellulose, 132.  
 " Forms of, 76.  
 " influence of antiseptics, 87.

Bacteria, influence of light, 86.  
 " " pressure, 80.  
 " " temperature, 85.  
 " " vibration, 87.  
 " involution forms, 78.  
 iron, 162.  
 lactic acid, 106.  
 Migula's system of, 89.  
 Nitrifying, 164.  
 nutrition, 81.  
 organs of motion, 6, 83.  
 Phosphorescent, 80.  
 Propagation of, 83.  
 Slime-forming, 142.  
 Spore-forming, 84.  
 Sulphur, 164.  
 with diastatic enzymes, 153.  
 " inverting enzymes, 151.  
 " proteolytic enzymes, 153.

Bacteriocytes, 80.

Bacterium aceti, 82, 95.  
 " *aceticum rosaceum*, 104.  
 " *acetigenum*, 101.  
 " *acetosum*, 101.  
 " *ascendens*, 102.  
 " *curvum*, 102.  
 " *gelatinosum betæ*, 150.  
 " *gummosum*, 143.  
 " *industrium*, 101.  
 " *Kützingianum*, 96.  
 " *lactis acidii*, 107, 109, 110, 112, 124.  
 " " *erythrogenes*, 115.  
 " " *longi*, 114.  
 " *mannitopum*, 123.  
 " *orleanense*, 103.  
 " *oxydans*, 100.  
 " *Pastorianum*, 96.  
 " *prodigiosum*, 111, 115.  
 " *Sacchari*, 151.  
 " *Schützenbachi*, 102.  
 " *soya*, 196.  
 " *synxanthum*, 115.  
 " *vermiforme*, 141.  
 " *vini acetati*, 103.  
 " *xylinoids*, 103.  
 " *xylinum*, 100.

Bacteroids, 80.

Beer, Belgian, 121.  
 " Cloudiness of, 9.  
 " Diseases in, 256.  
 " Filtration of, 16.  
 " Lactic acid in, 121.  
 " Sarcinae in, 155.  
 " Slime-forming bacteria in, 145.  
 " Sterilisation of, 23.  
 " " Weissbier," 121, 341.  
 " -wort, Action of yeasts on, 261.

Beer wort, Aération of, 280.  
 " " Production of acid in, 263.  
 " " " alcohol in, 261.  
 " " " glycerine in, 261.  
*Beggatooa alba*, 164.  
 Biological examination of air, 59.  
 " " ice, 71.  
 " " water, 69.  
 " " relationships of yeasts, 270.  
*Blästoderma salmonicolor*, 398.  
*Botrytis cinerea*, 176, 183.  
 Böttcher's moist chamber, 12, 44, 51.  
 Bottom fermentation yeasts, 283, 285, 311, 318, 320, 323.  
 Bouquet in wine, 265.  
 Brewery yeasts, 317.  
 Broth, Nutritive, 41.  
 Butter, Abnormal, 115.  
 " *Torula* yeasts in, 400.  
 Butyric acid bacteria, 125.

## C

**CALCIUM**, 172.  
 bisulphite, 34.  
 Carbohydrates in fungi, 182.  
 " Reaction of yeasts with, 238.  
 Carbolic acid, 28.  
 Carbon, 173.  
*Carlsberg* bottom yeast I, 261, 281, 320, 323.  
 " " 11, 261, 284, 320, 323.  
 " flask, 39.  
 Carragheen moss, 42.  
 Cell nucleus, 5, 300.  
 " wall, 300.  
 Cellulose, Bacteria-fermenting, 132.  
*Chalara mycoides*, 217.  
*Chamberland* flask, 37.  
 Cheese, Abnormal, 115.  
 " Ripening of, 110.  
 " *Sarcinae* in, 154.  
 " *Torula* yeasts in, 400.  
 Chemical constituents of fungi, 178.  
*Chlamydomyces Oryzae*, 205.  
*Chlamydospore*, 201.  
*Chloride* of lime, 32.  
 Chlorine, 27.  
 Chloroform, 27.  
 Cilia, 6, 83.  
 Citric acid from citromyces, 190.  
*Citromyces*, 190.  
 " " gläber, 190.  
 " " *Pfefferianus*, 190.  
*Cladosporium butyri*, 115.

*Cladosporium herbarium*, 221.  
*Cladotrichia dichotoma*, 162.  
*Clostridium butyricum*, 127, 129.  
 " " gelatinosum, 150.  
 " " *Pastorianum*, 131.  
 Cloudiness in beer, 9.  
 Cohn's nutritive fluid, 41.  
 Colonies, Giant, 43.  
 Conidia, 169.  
 " in moulds, 169.  
 Cream, Souring of, 116.  
*Crenothrix Kühniana*, 162.  
 Crystalloids in moulds, 167.  
 Cultures, Drop, 53.  
 " Indian ink point, 53.  
 " Liquid, 40.  
 " Plate-, 42.  
 " Stab-, 43.  
 " Streak-, 42.  
 " on gypsum blocks, 303, 311.  
 " on solid substrata, 297.

## D

**DEMATIUM pullulans**, 218.  
 " on grapes, 271.  
*Dextrose*, Yeast fermenting, 259, 267.  
 Diseases in beer, 245, 256.  
 Disinfectants, 24.  
 " Testing power of, 25.  
 Disinfection in practice, 31.  
*Dispora caecistica*, 136.  
 Distillery mash, 118.  
 " yeasts, 342.  
 Drop cultures, 53.  
 Drying methods, 5.

## E

**ELECTRICITY**, Influence on fungi, 177.  
*Endomyces decipiens*, 306.  
*Endotryptase* in yeast, 250, 256.  
 Enzymes, 180.  
 " in *Penicillium*, 190.  
 " of acetic acid fermentation, 94.  
 " of bacteria, 151.  
 " of lactic acid fermentation, 108.  
 " of yeast, 255.  
 " Proteolytic, 153, 250, 256.  
 " Reducing, 256.  
*Erysiphe* *Tuckeri*, 222.  
*Ether*, 27.  
 " as disinfectant, 28.  
 " as alcohol, 267.

Eurotium aspergillus glaucus, 193.  
Exosporium, 189.

## F

FATTY oils in moulds, 167.  
Fermentation, Products of alcoholic, 267.  
    Theories of, 229.  
Fermentative power of mucor, 208.  
    "    "    yeast, 270.  
    "    "    juice, 251.  
Film-formation, yeasts, 292.  
Filter, Cotton wool, 233.  
    "    Nordtmeyer, 15.  
    "    Pasteur-Chamberland, 15.  
Filtration of air, 17, 63.  
    "    beer, 16.  
    "    milk, 17.  
    "    water, 18.

Fischer's nutritive fluid, 41.  
Flagella, 6, 83.  
Flasks, 36.  
    "    Carlsberg, 39.  
    "    Chamberlain, 37.  
    "    Freudenreich, 37.  
    "    Hansen, 38, 419.  
    "    Jørgensen, 38, 419.  
    "    Pasteur, 36.

Foodstuffs, Inorganic, for yeasts, 226.  
    "    Organic, for yeasts, 227.

Formaldehyde, 27, 32.

Formalin, 32.

Formic acid, 268.

Freudenreich flask, 37.

"Frobbeg" yeast, 325.

Fructose, Yeast fermentin g, 267.

Fumago, 222.

Fungi, Carbohydrates in, 182.

    "    Chemical constituents of, 178.  
    "    influence of antisepsics, 178.  
    "    "    electricity, 177.  
    "    "    light, 176.  
    "    "    pressure, 177.  
    "    "    temperature, 175.

    "    "    vibration, 177.  
    "    "    nutritive, physiology of, 171.

Fusarium, 217.

## G

GELATINE, 42, 50.  
Gemmæ, 201.  
Giant colonies, 43.  
Ginger-beer plant, 140.

Glucose, Yeast-fermenting, 259, 267.  
Glycase, 247.  
Glycerine produced during fermentation, 253, 264, 267.  
Glycogen in yeast cells, 8, 227, 302.  
Gonidia in Crenothrix, 85.  
Granulæ in yeast cell, 9, 301.  
Granulobacter, 131, 135.  
    "    saccharo-butyricum, 126, 131.

Grapes, attacked by Botrytis, 186.  
    "    "    Oidium Tuckeri,  
    "    "    222.  
    "    "    Penicillium, 189.  
    "    "    Peronospora viticola, 223.  
    "    "    Dematium pululans on, 271.

Gypsum blocks, Cultures on, 303, 311.

## H

Hæmatimeter, 55.  
Hansen flask, 38, 419.  
Hops, Spontaneous heating of, 132.  
Hydrofluoric acid and fluorides, 27, 32, 46.  
Hydrogen fermentation, 133.  
    "    peroxide, 27, 35.

## I

Ice, Biological examination of, 71.  
Invertase in bacteria, 151.  
    "    Monilia, 213.  
    "    yeast, 246, 255.  
Involution forms, Bacteria, 78.  
Iron, 172.  
    "    bacteria, 162.  
Isomaltose, 260.

## J

JOHANNISBERG, I, 244.  
    "    II, 284, 286, 344.  
    "    "    Spores of, 303.  
Jørgensen's flask, 38, 419.  
    "    "    moist chamber, 51.

## K

KEPHIR, 135.  
Koji, 195.  
Koumiss, 138.

## L

Lactase in yeast, 248, 255.  
 Lactic acid, 268.  
 •, bacteria, 106.  
 " in the brewery, 121.  
 " " distillery, 118.  
 " " leaven, 124.  
 " " preserved foods, 125.  
 " " wine, 123.  
 Lactobacillus caucasicus, 136.  
 fermentum, 121.  
 Lactomyces inflans casei grana, 400.  
 Lactose, Torula yeasts fermenting, 399.  
 Levulose, Yeast fermenting, 267.  
 Leaven, Lactic acid in, 124.  
 Leben, 139, 413.  
 Leptothrix ochracea, 162.  
 Leuconostoc mesenterioides, 79, 148.  
 Levure caseeux, 283.  
 " de sel (a), 365.  
 " (b), 394.  
 " (γ), 394.  
 Light, influence on bacteria, 86.  
 " fungi, 176.  
 Lime, 27.  
 " Milk of, 35.

## M

Magnesium, 172.  
 Maltase, 247.  
 " in yeast, 255.  
 Maltose, Yeast fermenting, 260, 267.  
 Mash, Distilled, 118.  
 Yeast, 119.  
 " Maya," 140.  
 Mazun, 139.  
 Torula yeasts in, 401.  
 Melibiose i. yeast, 255.  
 Melibiose, 247.  
 Membrane of moulds, 167.  
 Mercuric chloride, 26.  
 Methane fermentation, 133.  
 Methyl alcohol, 268.  
 Micro-biological research, 10.  
 Micro-chemical examination, 8.  
 Micrococcus acidi levolactic, 110.  
 amaricans, 156.  
 " Billrothi, 150.  
 candidus, 156.  
 carneus, 156.  
 casei amari, 116, 156.  
 " liquefaciens, 111.  
 " cinnabarium, 156.  
 concentricus, 156.

Micrococcus flavus, 156.  
 " Freudenreichii, 114.  
 " gummosus, 144.  
 " lacticus, 156.  
 " luteus, 156.  
 " malolacticus, 124.  
 " uree, 156.

Microscope, 1.  
 Microscopical preparations, 1.

Mikrosol, 34.

Milk, Abnormal, 113.  
 " Filtration of, 17.  
 " Peptonised, 41.  
 " Sarcine in, 155.  
 " Slime-forming bacteria in, 145.  
 " Sterilisation of, 22.  
 " Torula yeasts in, 399.

Moist chamber, Böttcher's, 12, 51.  
 " Jörgensen's, 51.  
 " Ranvier's, 11.

Molasses, Slime-forming bacteria in, 148.

Monilia, 210.  
 " candida, 210, 213, 259.  
 " Invertase in, 213.  
 " sitophila, 214.

Montanin, 33.

Morphology of yeast cells, 290.  
 Moulds, 165.

Conidia in, 169.  
 " Crystalloids in, 167.  
 " Fatty oils in, 167.  
 " Membrane of, 167.  
 " Nucleus of, 167.  
 " Protoplasm in, 167.  
 " Spherical yeast, 168.  
 " Sporangium, 169.  
 " Spores of, 169, 170.  
 " Zygospores, 169.

Mucor, 197.  
 alpinus, 202, 207.  
 " alternans, 202.  
 " circinelloides, 202, 209.  
 " erectus, 202, 209.  
 " Fermentative power of, 209.  
 " javanicus, 204, 209.  
 " Mucedo, 199, 209.  
 " neglectus, 207.  
 " Praini, 204.  
 " pyriformis, 210.  
 " racemosus, 201, 202, 207, 209.  
 " Rouxii, 203, 209.  
 " spinosus, 202, 209.  
 " stolonifer, 204.

Must, see Wine must.

Mycoderma cerevisiae, 258, 407.  
 humuli, 398.  
 " rubrum, 398.

Mycoderma vini, 407.  
Myxo-bacteria, 79.

## N

NITRIFYING bacteria, 164.  
Nitrogen, 172.  
Nucleus of moulds, 167.  
Nutrition of yeasts, 225.  
Nutritive broth, 41.  
    " fluid, Cohn's, 41.  
    " " Fischer's, 41.  
    " " Pasteur's, 40.  
    " " Raulin's, 42.  
    " " Voges and Proskauer's,  
        41.  
    " substrata, 40.

## O

OBDIUM lactis, 106, 113, 115, 214.  
    Tuckeri, 222.  
Oil globules in yeast cells, 9.  
Ozone, 27, 35.

## P

PAPIN's digester, 15.  
Paraplectrum foetidum, 112, 131.  
Parasites, 81.  
Pasteur flasks, 36.  
Pasteur's nutritive fluid, 40.  
Pasteurisation, 21.  
Pediococcus acedulefaciens, 158.  
    " acidi lactic, 119.  
    " cerevisiae, 155.  
    " damnosus, 157.  
    " odoris mellisimilis, 157.  
    " perniciosus, 157.  
    " sarcinaformis, 156.  
    " viscosus, 145.  
Penicillium album, 113, 190.  
    " Camembert, 189.  
    " candidum, 113, 190.  
    " Enzymes in, 190.  
    " glaucum, 113, 175, 187, 190.  
    " italicum, 190.  
    " luteum, 190.  
    " olivaceum, 190.  
    " Roquefort, 189.  
Pronostpora viticola, 223.  
Petri dishes, 50.  
Phenol, 28.  
Phosphorus, 172.  
Photographs, Micro-, 7.

Phycomyces nitens, 207.  
Pichia californica, 367.  
    " farinosa, 368.  
    " membranæfaciens L, 366.  
    " " II, 367.  
    " " III, 367.  
    " Radaisii, 368.  
    " Tamarindorum, 367.  
    " taurica, 367.  
Plate-cultures, 42, 50, 53.  
" Podkvassa," 140.  
Point cultures, Indian ink, 53.  
Poisonous substances in fungi, 182.  
Poisons, influence of minute doses, 29, 174.  
Potassium, 172.  
Preserved foods, Lactic acid in, 125.  
Pressed yeast, 342.  
Pressure, influence on bacteria, 87.  
    fungi, 177.  
Products of alcoholic fermentation, 267.  
Propagating apparatus, Bergh and Jørgensen, 417.  
    " Hansen and Kühle,  
        415.  
Proteolytic enzymes, 153, 250, 256.  
Proteus vulgaris, 151.  
Protoplasm in moulds, 167.  
Pure culture, Despatch of, 419.  
    " dilution methods, 47.  
    " physiological methods, 44.  
    " Preparation of, 43.  
Pure culture of yeast on large scale, 414.  
Pyritic, 34.

## R

RANVIER's moist chamber, 11.  
Raulin's nutritive fluid, 42.  
Reducing enzymes, 256.  
Rennet, 111.  
Resting cells, Yeast, 295.  
Rhizopus japonicus, 205, 208, 210.  
    " nigricans, 204.  
    " Oryzæ, 205, 210.  
    " Tamari, 208.  
    " tonkinensis, 206, 210.

## S

" SAAZ" yeast, 325.  
Saccharobacillus Pastorius, 121.  
Saccharomyces acidi lactic, 370.  
    " anomalous, 278, 368  
    " " in beer, 257.  
    " " Spores of, 306.  
    " " in soil, 276.

*Saccharomyces apiculatus*, 258, 402.  
 „ „ „ *Habitat* of, 271,  
 „ „ „ 275.  
 „ „ „ *aquifolii*, 358.  
 „ „ „ *Bailii*, 363.  
 „ „ „ *Batatae*, 197, 359.  
 „ „ „ *Bayanus*, 356.  
 „ „ „ *brassicæ*, I., II., III.,  
 „ „ „ 395.  
 „ „ „ *Carlsbergensis*, 323.  
 „ „ „ *cartilaginosus*, 359.  
 „ „ „ *cerevisiae* I., 258, 277,  
 „ „ „ 285, 290, 293,  
 „ „ „ 326.  
 „ „ „ „ *Spores* of, 304,  
 „ „ „ „ 308.  
 „ „ „ Classification of, 314.  
 „ „ „ *Comesii*, 377.  
 „ „ „ *ellipsoideus* I., 258, 263,  
 „ „ „ 290, 294,  
 „ „ „ 351.  
 „ „ „ „ *Spores* of, 308.  
 „ „ „ „ II., 258, 263,  
 „ „ „ „ 277, 286, 290,  
 „ „ „ „ 294, 354.  
 „ „ „ „ in beer, 257,  
 „ „ „ „ 312.  
 „ „ „ „ *Spores* of, 308.  
 „ „ „ *exiguus*, 258, 262, 361.  
 „ „ „ *flava lactis*, 365.  
 „ „ „ *fœtidus* I. in beer, 258.  
 „ „ „ *fragilis*, 371.  
 „ „ „ *guttulatus*, 383.  
 „ „ „ *Hansenii*, 366.  
 „ „ „ *hyalosporus*, 363.  
 „ „ „ *ilicis*, 358.  
 „ „ „ *intermedius*, 347.  
 „ „ „ *Johannisberg* I., 344.  
 „ „ „ „ II., 284, 286,  
 „ „ „ „ 344.  
 „ „ „ „ II., *Spores* of,  
 „ „ „ „ 306.  
 „ „ „ *Jörgensenii*, 362.  
 „ „ „ *Kephr*, 400.  
 „ „ „ *Kreuznach*, 344.  
 „ „ „ *levure de sel* (a), 365.  
 „ „ „ *Logos*, 356.  
 „ „ „ *Ludwigii*, 258, 278, 281,  
 „ „ „ „ 298, 301, 306,  
 „ „ „ „ 374.  
 „ „ „ „ *Spores* of, 306.  
 „ „ „ *mali* *Duclaux*, 365.  
 „ „ „ „ *Risler*, 360.  
 „ „ „ „ *Marxianus*, 258, 278,  
 „ „ „ „ 298, 360.  
 „ „ „ „ *mellacci*, 381.  
 „ „ „ „ *membranafaciens*, 258,  
 „ „ „ „ 278, 297, 366.

*Saccharomyces membranafaciens* in soil,  
 „ „ „ 276.  
 „ „ „ minor, 366.  
 „ „ „ *Monacensis*, 323.  
 „ „ „ *Mülheim*, 344.  
 „ „ „ *multisporus*, 360.  
 „ „ „ *octosporus*, 282, 301,  
 „ „ „ „ 377.  
 „ „ „ *Pastorianus* I., 258, 263,  
 „ „ „ „ 277, 282, 284,  
 „ „ „ „ 290, 294, 345.  
 „ „ „ „ in beer, 257,  
 „ „ „ „ 312.  
 „ „ „ „ *Spores* of, 308.  
 „ „ „ *Pastorianus* II., 258,  
 „ „ „ „ 263, 277, 290,  
 „ „ „ „ 294, 347.  
 „ „ „ „ *Spores* of, 308.  
 „ „ „ *Pastorianus* III., 258,  
 „ „ „ „ 263, 277, 290,  
 „ „ „ „ 294, 349.  
 „ „ „ „ in beer, 257,  
 „ „ „ „ 312.  
 „ „ „ „ *Spores* of, 308.  
 „ „ „ *Piesport*, 344.  
 „ „ „ *pinophitorus* *enervans*,  
 „ „ „ „ 392.  
 „ „ „ „ „ *melodus*, 392.  
 „ „ „ *Pombe*, 380.  
 „ „ „ *pyriformis*, 141, 358.  
 „ „ „ *Rouxii*, 364.  
 „ „ „ *Saké*, 359.  
 „ „ „ *Saturnus*, 370.  
 „ „ „ *Soya*, 196, 364.  
 „ „ „ *thermuntitonum*, 357.  
 „ „ „ *turbidans*, 354.  
 „ „ „ *Tyrocola*, 400.  
 „ „ „ *unisporus*, 365.  
 „ „ „ *validus*, 349.  
 „ „ „ *Vordermanni*, 358.  
 „ „ „ *Walporzheim* I., 344.  
 „ „ „ *Willianus*, 355.  
 „ „ „ *Zoppii*, 362.  
 „ „ „ *Saccharomyces* *Ludwigii*, 374.  
 „ „ „ *Saccharomycopsis* *capsularis*, 384.  
 „ „ „ „ *guttulatus*, 383.  
 „ „ „ „ „ *Saccharose*, Yeast fermenting, 259,  
 „ „ „ „ „ 267.  
 „ „ „ „ *Sachsia* *suaveolens*, 214.  
 „ „ „ „ *Saké*, 195.  
 „ „ „ „ *Salicylic acid*, 35.  
 „ „ „ „ *Salt yeasts*, 394.  
 „ „ „ „ *Saprophytes*, 81.  
 „ „ „ „ *Sarcinae*, 153.  
 „ „ „ „ „ *acidificans*, 154.  
 „ „ „ „ „ *alutacea*, 154.  
 „ „ „ „ „ *aurantiaca*, 154.  
 „ „ „ „ „ *butyrica*, 154.

Sarcinæ, *candida*, 154.  
 " *casei*, 154.  
 " *flava*, 154.  
 " *fusca*, 155.  
 " *Hamaguchiæ*, 196.  
 " *lutea*, 154.  
 " *maxima*, 155.  
 " *mobilis*, 155.  
 " *nosacea*, 155.  
 " *rosea*, 115.  
 " *rubra*, 155.  
 Sarcinæ in beer, 155.  
 " cheese, 154.  
 " milk, 155.  
 Schizosaccharomyces *comesii*, 377.  
 " *mellacei*, 286, 381.  
 " *octosporus*, 377.  
 " *Pombe*, 286, 380.  
 Sclerotia, 168.  
 Sclerotinia *Fuckeliana*, 183.  
 Sclerotium of *botrytis*, 185.  
 Semiclostridium *commune*, 150.  
 Slime-forming bacteria, 142.  
 " in beer, 145.  
 " in milk, 145.  
 " in molasses, 148.  
 " in wine, 142.  
 Soda, 27, 35.  
 Sodium hypochlorite, 27.  
 sulphite, 27.  
 Soja, 196.  
 Solid substrata, Cultures on, 297.  
 Sphaerotilus *dichotoma*, 162.  
 Spherical yeast, moulds, 168.  
 Spontaneous generation, 232.  
 Sporangia, 169.  
 Spore-formation, 281, 304.  
 Spores, Bacteria, 84  
 " moulds, 169, 170.  
 " of Saccharomyces *anomalus*, 306  
 " " *cerevisiae* I, 304,  
 " " 308.  
 " " *ellipsoideus* I, 308.  
 " " II, 308.  
 " " *Johannisberg* II,  
 " " 306.  
 " " *Ludwigii*, 306.  
 " " *Pastorianus* I, 308.  
 " " " II, 308.  
 " " " III, 308.  
 Sporodinia *grandis*, 207.  
 Stab-cultures, 43.  
 Staining methods, 5.  
 Steam, Disinfection by, 14, 35.  
 Sterigma, 187.  
 Sterigmatocystis *niger*, 194.  
 Sterilisation, 13.  
 " Discontinuous, 20.

Sterilisation, Fractional, 20.  
 " of air, 23.  
 " of beer, 23.  
 " of glass and metal objects,  
 " 14.  
 " of liquid and solid sub-  
 strata, 15.  
 " of milk, 22.  
 Stimulating action of antiseptics, 20,  
 174.  
 Streak-cultures, 42.  
 Streptococcus *hollandicus*, 114.  
 " *lacticus*, 109, 112, 116.  
 " *mesenterioides*, 148.  
 Structure of yeast cells, 293.  
 Substrata, Nutritive, 40.  
 Succinic acid, 268.  
 Sugars, Fermentation of, 267.  
 " Synthesis of, 246.  
 " Synthetically prepared, 247.  
 " Yeasts fermenting certain, 250.  
 Sulphur, 172.  
 " bacteria, 164.  
 Sulphuric acid, 35.  
 Sulphurous acid and sulphites, 27, 34.

T

TAO-TJIUNG, 197.  
 Tane-Koji, 195.  
 Temperature, influence on bacteria, 83.  
 " " fungi, 175.  
 " " yeast cells, 277.  
 Thamnidium *elegans*, 207.  
 Theories of fermentation, 229.  
 Thermobacterium *Zeidleri*, 100.  
 Thymol, 27.  
 Tobacco, Fermentation of, 160.  
 Toluol, 27.  
 Top-fermentation yeasts, 311, 319, 329.  
 Pure cultures of, 341.  
 Torula *a* = *Torula Holmii*, 388.  
 " *b* = " *mucilaginosa*, 395.  
 " *c* = " *cinnabarinæ*, 396.  
 " *amara*, 401.  
 " *colliculosa*, 393.  
 " *epizoa*, 394.  
 " *novæ carlsbergiæ*, 260, 288.  
 " in beer, 257.  
 " yeasts fermenting lactose, 399.

Torulas, 259, 385.  
 Turbidity in beer, 9.  
 Tyrothrix, 112.

U

ULTRA-MICROSCOPE, 8.

## V

VACUOLES in yeast cells, 301.  
 Variations in the *Saccharomyces*, 280.  
 " in yeasts occurring in practice, 288.  
 VIBRATION, influence on bacteria, 87.  
 " " fungi, 177.  
 Vihegar process, Orléans, 93.  
 " " Pasteur's, 93.  
 " " " Quick," 93.  
 Vitalistic theory of fermentation, 235.  
 Voges and Proskauer's nutritive fluid, 41.

## W

WATER, Biological examination of, 69.  
 " " examination of, by Hansen, 71.  
 " " Holm, 69.  
 " " Jørgensen, 70.  
 " " Lindner, 74.  
 " " Wichtmann, 73.  
 Filtration of, 18.  
 Weissbier," 121, 341.  
*Willa anomala*, 368.  
*W. Saturnus*, 370.  
 Vine, Lactic acid in, 123.  
 Production of acid in, 265.  
 " alcohol in, 265.  
 " bouquet in, 265.  
 " glycerine in, 264.  
 " Pure cultures of yeasts in, 266.  
 Slime-forming bacteria in, 142.  
 " must, Action of yeasts on, 263.  
 " yeast in soil, 274.  
 yeasts, 343.  
 Wort, Aération of, 280.  
 " see *Beer-wort*.

## Y

YAM brandy, 197.

Yeast, Analysis of, 311.  
 " Biological relationships of, 270.  
 " Fermenting power of, 270.  
 " Pure culture of, on large scale 414.

## Yeast cells, Counting, 55.

" effect of nutritive fluid, 279.  
 " " temperature, 277.  
 " film-formation, 292.  
 " Resting, 295.  
 " spore-formation, 302.  
 " Structure of, 298.

## Yeast deposits, 290.

Yeast juice, Fermentation of, 252.  
 Preparation of, 248.

## Yeast mash, 119.

Yeast propagating apparatus, Bergh and Jørgensen, 417.  
 " Hansen and Küller, 415.

## Yeasts, 225.

" Brewery, 317.  
 " Culture, 317.  
 " Distillery, 342.  
 " habitat in soil, 274.  
 " Nutrition of, 225.  
 " Pressed, 342.  
 " Top-fermentation species, 311, 319, 329.  
 " Wine, 343.

## Yoghourt, 140.

## Z

ZOOGLOEA, 79.  
*Zygosaccharomyces Barkeri*, 372.  
 Zygospores, 169.  
 Zymase, 181, 249.  
 " Properties of, 254.







